には、オアフ島での採集において、我々が *Sida* 属植物と共に採集した試料 (CMH type B) と葉や分果が類似しているもの (SCA-4, 5, BSS-3, 4b) があった。今回、オアフ島で採集した 6 個体は、いずれも半径 1 m 以内に自生しており、このような状況の中、正確に *Sida* 属植物のみを採集する事は困難であると思われる。このことから、海外の生薬生産及び原料植物の採集の現場においても、*Sida* 属植物の中に、別のアオイ科植物が自生していた場合、同様のことが生じると想像され、このことが、*Sida* 属植物製品に別のアオイ科植物が混入する原因の一つであると考えられる。

また、混入していた植物の中には、*Achyranthes* 属植物の様に、日局ゴシツの基原植物として、食薬区分において「専ら医薬品としてもちいる成分本質（原材料）」とされているものも見受けられた。今回、検出された *Achyranthes* 属植物は、葉であり、「非医」相当のため、直ちに健康被害が危惧されるものとは考えにくいが、アオイ科を中心に非常に多種の植物が *Sida* 属植物製品の原料となっている実体が明らかになった。本研究は、*Sida* 属植物中のアルカロイド含有種の範囲を調べることを目的に開始されたが、*Sida* 属植物のアルカロイドによる健康被害の発生とは別に、原料植物の誤同定に起因した有毒植物の混入による事故の発生も懸念された。我々は、これまでの研究において、薬用植物を原料に用いた健康食品等に、故意あるいは非意図的な別種植物の混入例を多数、確認している[13-16]。今回の研究は、植物利用製品における、原料植物の同定の重要性を改めて示す結果となった。

V　謝辞

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VI　参考文献

1) Mabberley, D. J., "Mabberley's plant-book: A portable dictionary of plants, their classification and uses", 3rd Ed., New York, USA, Cambridge University Press, 2008, p. 795. (ISBN 978-0-521-82071-4)
2) Aguilar, J. F., Fryxell, P. A., Jansen, R. K.: Phylogenetic relationships and classification of the Sida generic alliance (Malvaceae) based on nrDNA ITS evidence, Syst. Bot., 28, 352-364 (2003).
3) Tate, J. A., Aguilar, J. F., Wagstaff, S. J., Duke, J. C. L., Slotta, T. A. B., Simpson, B. B.: Phylogenetic relationships within the tribe Malveae (Malvaceae, subfamily Malvoideae) as inferred from ITS sequece data, Am. J. Bot., 92, 584-602 (2005).
4) Kapoor, L. D., "Handbook of Ayurvedic Medicinal Plants", Florida, USA, CRC Press, 1990, p. 303. (ISBN 0-8493-0559-4)
5) Khatoon, S., Srivastava, M., Rawat, A. K. S., Mehrotra, S.: HPTLC method for chemical standardization of *Sida* species and estimation of the alkaloid ephedrine, J. Planar Chromatogr., 18, 364-367 (2005).
6) "Chuyaku-Daijiten", Shanghai Kagakugijutsu Shuppansha and Shogakukan ed., Tokyo, Japan, Shogakukan, 1985, p. 115-119. (ISBN 4-09-583001-8)
7) "Flora of Kanagawa 2001", The Flora-Kanagawa Association ed., Odawara, Japan, the Kanagawa Prefectural Museum of Natural History, 2001, pp. 995-996.
8) Regulatory Control of Unapproved/Unlicensed Drugs (Pharmaceutical Affairs Bureau Notification No. 476 of Jun. 1, 1971; final major revision, Pharmaceutical and Food Safety Bureau Notification No. 0417001 of Apr. 17, 2007; final revision, Pharmaceutical and Food Safety Bureau Notification No. 0220001 of Feb. 20, 2009), The Ministry of Health, Labour and Welfare, Japan.
9) Prakash, A., Varma, R. K., Ghosal, S.: Alkaloidal constituents of *Sida acuta*, *S. humilis*, *S. rhombifolia* and *S. spinosa*, Planta Med., 43, 384-388 (1981).
10) White, T. J., Bruns, T., Lee, S., Taylor, J. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in Innis", M. A., Gelfand, D. H., Sninsky, J. J., White, T. J. ed., "PCR Protocols-A guide to methods and applications", San Diego, USA, Academic Press, 1990, pp. 315-322. (ISBN 0-12-372180-6)
11) Kimura M.: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences., J. Mol. Evol., 16, 111-120 (1980).
12) Saitou N., Nei M.: The neighbor-joining method: A new method for reconstructing phylogenetic trees., Mol. Biol. Evol., 4, 406-425 (1987).
13) Maruyama T., Sugimoto N., Kuroyanagi M., Kim I. H., Kamakura H., Kawasaki T., Fujita M., Shimada H., Yamamoto Y., Goda Y., Authentication and chemical study of Isodonis extract and Isodonis Herba, Chem. Pharm. Bull., 55, 1626-1630 (2007).
14) Maruyama T., Kamakura H., Miyai M., Komatsu K., Kawasaki T., Fujita M., Shimada H., Yamamoto Y., Shibata T., Goda Y., Authentication of the traditional medicinal plant *Eleutherococcus senticosus* by DNA and chemical analyses, Planta Med., 74, 787-789 (2008).
15) Goda Y., The safety of health foods and importance of their origin, Yakugaku Zasshi, 128, 837-838 (2008).
16) Kamakura H., Maruyama T., Sugimura K., Iida O., Goda Y., The source plants and the chemical composition of the passion flower products available as dietary supplements, Jpn. J. Food Chem. Safety, 17, 198-206 (2010).

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Quality Assurance of Natural Products: Miscellaneous Thoughts from the Fact That Shatavari Does Not Contain Alkaloid Asparagamines

天然物の品質保証ーシャタバリには、アスパラガミン類アルカロイドは含まれていないという事実から考えること

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国立医薬品食品衛生研究所生薬部では、所掌にはないが、国立医薬品食品衛生研究所のミッションの一つと考え「科学的な知見に基づく食薬区分」に関し厚生労働科学研究等で対応している。食薬区分とは、正確にいえば「無承認無許可医薬品の（薬事法による）指導取締りのための区分」であるが、申請のあった個々の成分・本質について「医薬品の範囲に関する基準」に基づいて、「専ら医薬品成分」であるかどうかを判断する行為ともいえる。厚生労働省の医薬局長通知である「医薬品の範囲に関する基準」では、食薬区分の判断基準を公開しており、成分本質のみで、「専ら医薬品」と判断されるものは、

1. 専ら医薬品として使用実態のある物（解熱鎮痛消炎剤、ホルモン、抗生物質、消化酵素等）
2-1. 毒性アルカロイド、毒性タンパク、毒薬劇薬指定成分に相当する成分を含むもの（食品衛生法で規制される、植物性自然毒、動物性自然毒を除く）
2-2. 麻薬、向精神薬及び覚醒剤様作用があるもの（類似化合物も含む）及びその原料植物
2-3. 指定医薬品または処方せん医薬品に相当する成分を含むものであって、保健衛生上医薬品として規制する必要性があるもの

のどれかでなくてはならない。従って、2-1に記載された、アルカロイドの含有は、専ら医薬品と判断するための重要な指標となる。

アユルヴェーダ生薬であるシャタバリは、*Asparagus racemosus*の根を基原とし、女性向けの栄養補助、強壮用の用途に使用されており、成分として植物エストロゲンと考えられるステロイドサポニン類や、フラボノイド類に加え、pyrrolo[1,2-a]azepine アルカロイドであるasparagamine類を含むことが報告されている。asparagamine類は、肝毒性のあるピロリジジンアルカロイドの一種であり、本化合物を含有する場合、これまでの慣例に従うと上記の2-1項に該当するため、「専ら医薬品成分」と判断されることになる。

他方、筆者らの分析[1,2]では、シャタバリ製品11検体からは、asparagamine A及び、pyrrolo[1,2-a]azepineアルカロイド類縁体は全く検出されなかった。そこで、本品及び*Asparagus racemosus*の根の標品について、遺伝子解析を行ったところ、シャタバリ製品の基原は、*A. racemosus*であることが明らかとなり、さらに、この事実は、これらの製品の化学分析、即ち、主要なステロイドサポニン shatavarin IVと他のステロイドサポニン類の検出パターンからも支持された。

これまでの報告によると、asparagamine類は、*A. racemosus*で最初に単離されたものの、*Stemona*属でも単離が報告されている。また、asparagamine Aが単離された*A. racemosus*より、同時にdihydrophenanthrene誘導体であるracemosol（=stemanthrene D）の単離も報告されているが、この化合物は*S. collinsae*をはじめいくつかの*Stemona*属植物に特有のスチルベンであることが示されている。さらに、*A. racemosus*と*Stemona*属植物の根は形態が非常に似ていることも報告されている。これらの点を鑑みると、*A. racemosus*からのasparagamine Aの単離の報告は、*Stemona*属植物を*A. racemosus*と誤同定した結果による可能性が非常に高いと考えられた。

そこで、これまで同植物より、asparagamine Aやpyrrolo[1,2-a]azepineアルカロイドを単離した複数の研究者に連絡をとり、単離原料が残っていないか問い合わせたが、現段階では、どの研究者からも実験材料が残っていない等の返事を頂き、アルカロイド類を単離した実験材料の基原の最終確認を行うことができなかった。

しかしながら、食薬区分の判断を行うには、前述した実験結果及び文献からの推定は非常に重要なものと考え、筆者らはシャタバリには、asparagamine類は含まれないという判断を前提に検討をすすめ、同品目は、「医薬品的効能効果を標榜しない限り医薬品と判断しない成分本質（原材料）リスト」（非医リスト）に収載されることになった。

筆者らは、従来より、天然物の品質保証の第一歩は、基原の正しい植物を使用することであるとの観点から、天然物製品の基原に関する研究を行ってきた。その結果、多くの健康食品で、正しい基原の植物が使用されていないことを明らかにしている。本誌においても、2007年の5月号で、「天然物の基原と品質」という内容で、特集号を組ませて頂いたことがある[3]。また、研究の概略について、2011年5月の食品化学学会の大会長

講演をはじめとして、いくつかの講演会で講演させて頂いた。しかし、今回の結果は、これまでと全く違うパターンであり、調査を行ったシャタバリ健康食品は、全て正しい基原の*A. racemosus*が使用されていた。しかしながら、食薬区分の判断の資料となる学術論文において、天然物化学の研究者が、成分の検索、構造決定を行った原材料について、基原植物の同定を正しく行わなかったことに起因した混乱であった。事実、asparagamine類をシャタバリから単離したという論文では、実験材料の同定や入手経路や保存方法について、全く記述がない論文も見受けられた。

筆者は、27年前「鶏血藤」と呼ばれる駆瘀血生薬からプロスタグランジン生合成阻害物質を単離、同定したが、学会のシンポジウムで発表の際、大阪大学の北川先生から、生薬の基原植物をしっかり同定したか問われて、それまで、その重要性について、全く認識していなかったことに気づかされた記憶がある。(恥ずかしい話である)

どうも、天然物化学の研究者は、面白い化合物の単離、同定にエネルギーを傾ける一方で、その原材料の基原についての確認がおざなりになり易い傾向があるように思う。生薬学は、そもそも正しい(同じ)基原の植物が使用された生薬をコンスタントに供給することを一つの目的とし、いわば一種の品質保証学として発達してきたわけであるが、生薬学から発達した天然物化学においても、そのルーツが品質保証学であったことを忘れないようにすることが肝要と考える。さらにいえば、薬学そのものが、医薬品や食品、生活関連物質の品質保証学としての要素をルーツにもった学問であって、創薬と、薬局や病院の薬剤師養成だけが、薬学の本質ではないと考える。

薬剤師でなければできない業務として「医薬品の製造管理責任者」があるのをご存じだろうか。また、食品衛生管理者は薬剤師であれば取れる資格である。薬学出身者や薬剤師が社会に期待される重要な役割として、医薬品や食品の品質保証、品質管理があることは間違いない。品質が保証され、管理されている医薬品や食品を使用できてこそ、初めて安心・安全な社会に生活することができる。

食薬区分の話から始まって、話がややそれてしまったが、国立医薬品食品衛生研究所生薬部では、引き続き、天然物の有効性と安全性、そして有効性と安全性を保証する品質に関連する研究を実施していきたいと考えている。

引用文献

1) Y. Kumeta, T. Maruyama, D. Wakana, H. Kamakura and Y. Goda, Chemical analysis reveals botanical origin of shatavari products and confirms of absence of alkaloid asparagamine A in *Asparagus racemosus*., *J. Nat. Med.*, on line available, DOI 10.1007/s11418-012-0669-4.
2) Y. Kumeta, T. Maruyama, D. Wakana, H. Kamakura and Y. Goda, Method for identifying botanical origin of shatavari product and its application for survey analysis of the products in Japanese market., *Jpn. J. Food Chem. Safety*, **18**, 163-167 (2011).
3) Y. Goda, The origin of natural products and their quality, *Foods and Food Ingredients Journal of Japan*, **212**, 343-344 (2007).

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ノ ー ト
Note

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Dapoxetine および flibanserin の LC-PDA-MS 分析

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LC-PDA-MS analysis of dapoxetine and flibanserin

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Abstract

Dapoxetine (1) and flibanserin (2) have pharmaceutical effects against premature ejaculation (PE) and hypoactive sexual desire disorder (HSDD), respectively. In international markets, these compounds have been reported as illegal additives in health foods for which tonic effects are implicitly indicated. Therefore, we performed LC-PDA-MS analysis in preparation for the distribution of illegal health foods containing these compounds in Japan. Compounds 1 and 2 were completely separated at r.t. 11.3 and 10.7 min, respectively, under the conditions described as the analytical method for udenafil by the Ministry of Health, Labour and Welfare, Japan. Their spectroscopic data (UV and MS) corresponded to the literature data. Subsequently, we added authentic dapoxetine and flibanserin to an extract of the food supplements with or without an ED treatment agent including sildenafil and tadarafil etc., and then these sample solutions were analyzed using the proposal method. As a result, each compound was completely separated on the UV and mass chromatograms. This study provides useful data for the surveillance of unapproved/unlicensed drugs in health food products.

Keywords : dapoxetine, flibanserin, LC-PDA-MS analysis
ダポキセチン、フリバンセリン、LC-PDA-MS 分析

I 緒言

近年、健康食品中への無承認無許可医薬品の含有が原因と思われる健康被害が多数発生している。痩身用を暗に標榜する健康食品では、食欲抑制剤およびその誘導体、下剤およびその作用を有する生薬を、強壮を暗に標榜する健康食品では、ED (Erectile Dysfunction) 治療薬およびその類似化合物 (Fig. 1) を、それぞれ意図的に加えた製品は、日本においても、この 10 年継続的に出現している。さらに、このような製品を摂取し、頭痛、嘔吐、動悸などの症状を訴える事例報告があり、重篤な場合には、死に至ったケースもある。

厚生労働省では、都道府県の協力を得ながら、「医薬品の範囲に関する基準」[1] に基づき、痩身や強壮を標榜する健康食品の実態調査を実施している。近年の傾向としては、店頭で入手できる製品からの医薬品成分の検出事例は少なくなっている。一方、インターネットを介して販売される強壮用製品

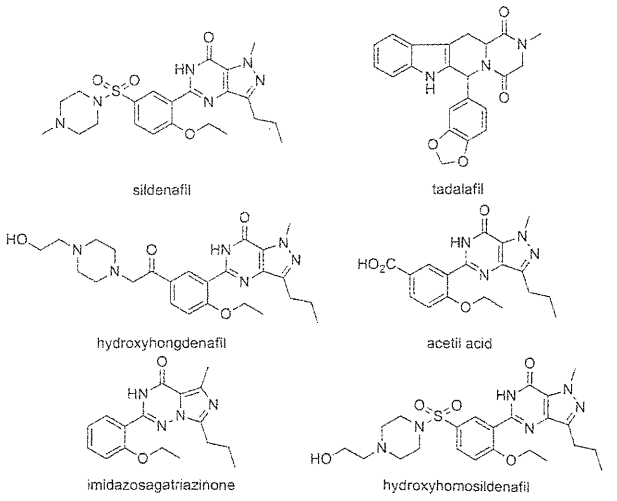

Fig. 1. Structures of several therapeutic agents for erectile dysfunction (ED) and their analogues

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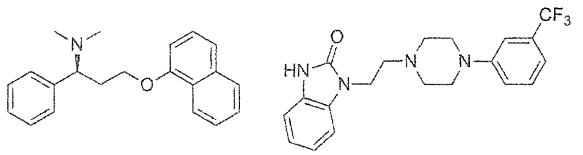
からは、依然としてED治療薬およびそれらの類縁体が検出されている[2-13, *1]。このうち、mutaprodenafilは、プロドラッグタイプの無承認医薬品成分であり、新しいタイプの成分として注目を浴びている。

近年、海外市場では、強壮を標榜する健康食品から、これまで主流だったED治療薬とは別に、早漏防止作用や性欲減退改善作用を有するdapoxetine (1) およびflibanserin (2) の流通が報告されている[14, 15] (Fig. 2)。化合物1は、選択的セロトニン再取込み阻害作用を有する化合物であり、早漏防止作用が認められていることから、医薬品としての開発が進められている[16]。化合物2は、閉経前女性の性欲減退に対する治療薬として開発が進められた化合物であり、第三相臨床試験まで治験が進んだが、目眩、吐き気、倦怠感などの副作用のため、米FDAにより、承認申請が却下されている[17]。インターネットの普及により、情報、流通のグローバル化が進む現在、海外での有害事例は多くの場合、日本国内でも発生することが予期される。

本研究では、1または2を含有する健康食品が日本の市場に流通した場合に備え、両化合物の標準品を入手し、各種機器分析データおよびLC-PDA-MS分析法をまとめたので報告する。



dapoxetine (1)　　flibanserin (2)

Fig. 2. Structures of dapoxetine (1) and flibanserin (2)

Ⅱ　方法

1. 実験材料

化合物1および2の標準品は、それぞれToronto Research Chemicals社およびAxon Medchem社より購入した。各標準品は、NMRおよびMS分析により構造を確認した。NMR測定装置として、JEOL ECA-800を使用した。MS測定装置として、イオン源DART-100（JEOL）に質量分析計AccuTOF JMS-100（JEOL）を連結したものを使用した。ED治療薬およびその類縁体を含有する健康食品は、次の4製品を用いた：I、三便宝；II、陰茎増大丸；III、HIDEYOSHIヒデヨシ；IV、壮三天。これらの製品は、当研究部の試験業務により、Table 1に示した各化合物を含有することが、既に確認されているものである。また、当該化合物を含有しないことが確認されていた1製品（V: EROTIC SENSE）をブランクとして分析試料に加えた。

Table 1. A list of food supplements that contain therapeutic agents for ED and their analogues as ingredients

Sample number	Product name	Ingredients
I	三便宝	Hydroxyhomosildenafil
	(Sambempo)	Tadalafil
II	陰茎増大丸	Sildenafil
	(Inkeizodaigan)	Tadalafil
III	HIDEYOSHI ヒデヨシ	Hydroxyhongdenafil
		Acetil acid
		Imidazosagatriazinone
IV	壮三天	Sildenafil
	(Sosanten)	Tadalafil
V	EROTIC SENSE	Not detected

2. 試料調製

化合物1および2について、1 mgをそれぞれメタノール1 mLに溶解し、標準溶液Aとした。また、標準溶液Aをメタノールにより100倍希釈したものを標準溶液Bとした。ED治療薬およびその類縁体を含有する健康食品は、それぞれ200 mgに1%ギ酸溶液/アセトニトリル（1/4）2 mLを加え、超音波下5分間抽出した。抽出液の上清1 mLに移動相A液1 mLを加え、膜ろ過を行った。これらの溶液240 μLに、標準溶液Aを、各30 μL、スパイクし、分析用試料溶液とした。

3. LC-PDA-MS分析

厚生労働省の通知を参考に以下の条件で行った[18]。LC-PDA-MSは島津製作所製ポンプ、LC-20AD、ダイオードアレイ検出器、SPD-M20A、質量分析計、LCMS-2020を用いた。また、1および2の標準溶液はスキャンモード、各健康食品製品は、SIMモードにより分析を行った。

3-1. LC条件

カラム：Inertsil ODS-3（2.1 × 150 mm、5 μm、GL Sciences）、移動相A：アセトニトリル/5 mMギ酸アンモニウム緩衝液（pH 3.5）25/75、移動相B：アセトニトリル、グラジエント条件：A/B 100/0（0 - 3 min）- 3%/min - 70/30（13 - 30 min）、流速：0.3 mL/min、カラム温度：40 °C、注入量：1 μL、検出：ダイオードアレイ検出器（190 - 800 nm；モニター波長290 nm）。

3-2. MS条件

イオン化法：ESIポジティブモード、ネブライザーガス流量：1.5 L/min、ドライイングガス流量：10 L/min、DL温度：250 °C、ヒートブロック温度：200 °C、検出器電圧：1.20 kV、インターフェイス電圧：4.5 kV、インターフェイス電流：6.7 μA、DL電圧：0 V、測定質量電荷比：*m/z* 100 - 800（スキャンモード）；*m/z* 306、313、357、390、391、475、483、505（SIMモード）。

*1　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

Ⅲ 結果および考察

方法の部で示した条件において、1および2の標準溶液AをLC-PDA-MS分析した結果、それぞれ、保持時間11.3分および10.7分に溶出された。これらのUVスペクトルは既報の文献値と一致した[14, 15] (Fig. 3-a, b)。また、標準溶液Bを分析した結果、当該ピークのマススペクトルにおいて、各化合物から予想される分子量に由来する擬似分子イオンピークをベースピークとして検出した (Fig. 3-c, d)。

本分析法の有用性を確認するために、健康食品 (試料I－V) から調製した試料溶液に、各標準溶液Aを一定量添加し、標準溶液と同様に分析を行った。これらの健康食品製品の内、試料I－IVは、Table 1に示したED治療薬及びその類縁体を含有する製品であり、試料Vは、これらの化合物を含有しない製品である。結果をFigs. 4、5に示した。上記の実験条件において、1および2は、UVクロマトグラムおよびマスクロマトグラムで、良好な分離を示し、それぞれの化合物の同定が可能であった (Fig. 4)。試料II－Vについても UVおよびマスクロマトグラム、ともに良好な分離を示した (Fig. 5)。

両化合物とも、udenafilの分析方法として厚生労働省通知された分析条件[18]において、カラム担体に十分に保持され、分析が可能であることが確認された。また、UVクロマトグラムにおいて、両化合物のピークは完全分離した。最所ら[19]は、同様の分析法を用いて、ED治療薬およびその類似化合物13種について分析を行っている。その結果では、sildenafil

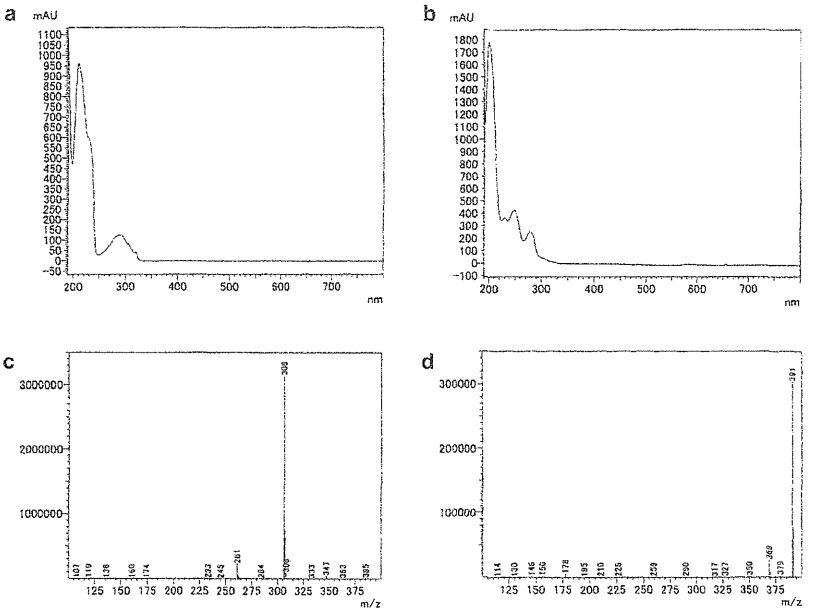


Fig. 3. UV and mass spectra of dapoxetine (1: a and c) and flibanserin (2: b and d)

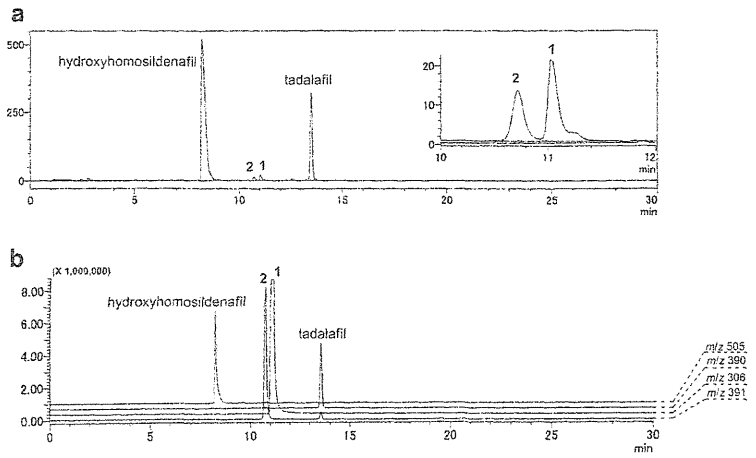


Fig. 4. UV at 290 nm (a) and mass (b) chromatograms of sample solution prepared from a food supplement (Sambempo) spiked with authentic dapoxetine (1) and flibanserin (2) on LC-PDA-MS analysis

Box in panel a indicates magnified UV chromatogram between 10 and 12 min.

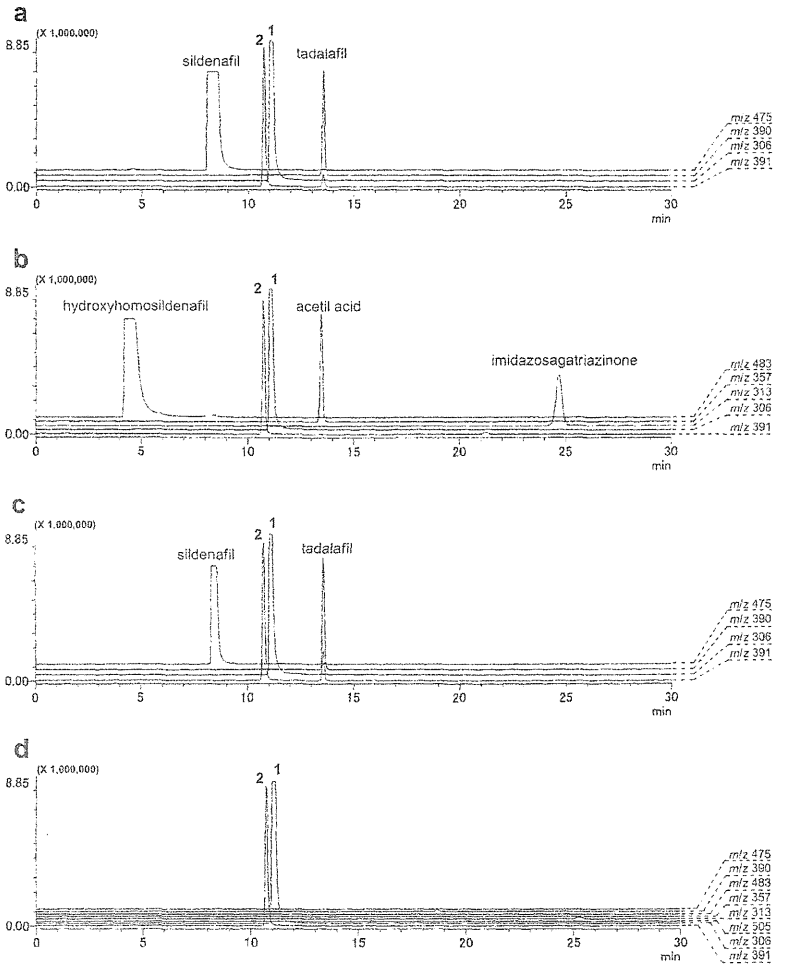


Fig. 5. Mass chromatograms of sample solution prepared from food supplements for tonicity spiked with authentic dapoxetine (1) and flibanserin (2) on LC-PDA-MS analysis
Panels a to d indicate data of Inkeizodaigan, HIDEYOSHI, Sosanten and EROTIC SENSE, respectively.

と tadalafil の間に、udenafil、aminotadalafil、thiodenafil の 3 化合物が溶出されている。一方で、Fig. 5 に示したように、1 および 2 も sildenafil と tadalafil の間に溶出された。したがって、強壮用健康食品に、1 および 2 が添加されたものが流通した場合、udenafil、aminotadalafil、thiodenafil の 3 化合物が共存していなければ、本分析法における当該ピークの UV スペクトルおよび MS から、添加物質の推定が容易に可能であると考えられた。

以上のことから、本分析法は 1 および 2 を含有する健康食品製品が流通した場合の監視業務に有用であり、迅速な対応が可能になると考えられた。

Ⅳ　結論

海外の建国食品市場に流通する製品から検出事例が報告された dapoxetine および flibanserin の標準品を購入し、各種機器分析データおよび分析法をまとめた。本報告で示した分析法は、両化合物が強壮用健康食品へ添加された際に分析をする上で有用な参考資料になると考えられる。

Ⅴ　謝辞

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Ⅵ　文献

1) Regulatory Control of Unapproved/Unlicensed Drugs (Pharmaceutical Affairs Bureau Notification No. 476 of Jun. 1, 1971; final revision, Pharmaceutical and Food Safety Bureau Notification No. 0120-3 of Jan. 23, 2012), The Ministry of Health, Labour and Welfare, Japan.
2) Takahashi, K., Hasegawa, T., Saijo, M., Nagata, T., Wakana, D., Goda, Y.: Identification of sildenafil analogues found in a dietary supplement. Annual Report of the Chiba Prefectural Institute of Public Health, 58, 55-60 (2009).

3) Demizu, Y., Wakana, D., Kamakura, H., Kurihara, M., Okuda, H., Goda, Y.: Identification of mutaprodenafil in a dietary supplement and its subsequent synthesis. Chem. Phram. Bull., 59, 1314-1316 (2011).
4) Kumasaka, K., Kawahara, N., Doi, K., Kojima, T., Goda, Y.: Determination of (*R*)-xanthoanthrafil, a phosphodiesterase-5 inhibitor, in a dietary supplement promoted for sexual enhancement. Chem. Pharm. Bull., 56, 227-230 (2008).
5) Goda, Y., Kamakura, H.: Erectile dysfunction drug ingredients and related compounds found in health foods and their adverse effects. Functional Food, 2, 198-202 (2008).
6) Uchiyama, N., Saisho, K., Kikura-Hanajiri, R., Haishima, Y., Goda, Y.: Determination of a new type of phosphodiesterase-5 inhibitor, thioquinapiperifil, in a dietary supplement promoted for sexual enhancement. Chem. Pharm. Bull., 56, 1331-1334 (2008).
7) Hasegawa, T., Takahashi, K., Saijo, M., Ishii, T., Nagata, T., Haishima, Y., Goda, Y., Kawahara N.: Isolation and structural elucidation of cyclopentynafil and N-octylnortadalafil found in a dietary supplement. Chem. Pharm. Bull., 57, 185-189 (2009).
8) Blok-Tip, L., Zomer, B., Bakker, F., Hartog, K. D., Hamzink, M., Ten Hove, J., Vredenbregt, M., De Kaste, D.: Structure elucidation of sildenafil analogues in hebal products. Food Addit. Contam., 21, 737-748 (2004).
9) Oh, S. S., Zou, P., Low, M. Y., Koh, H. L.: Detection of sildenafil analogues in herbal products for erectile dysfunction. J. Toxicol. Environ. Health A, 69, 1951-1958 (2006).
10) Reepmeyer, J. C., Woofruff, J.T.: Use of liquid chromatography-mass spectrometry and a hydrolytic techniwue for the detection and structure elucidation of a novel synthetic vardenafil designer drug added illegally to a "natural" herbal dietary supplement. J. Chromatogr. A, 1125, 67-75 (2006).
11) Choi, D. M., Park, S., Yoon, T. H., Jeong, H. K., Pyo, J. S., Park, J., Kim, D., Kwon, S. W.: Determination of analogs of sildenafil and vardenafil in foods by column liquid chromatography with a photodiode array detector, mass spectrometry, and nuclear magnetic resonance spectrometry. J. AOAC Int., 91, 580-588 (2008).
12) Zou, P., Hou, P., Oh, S. S., Chong, Y. M., Bloodworth, B. C., Low, M. Y., Koh, H. L.: Isolation and identification of thiohomosildenafil and thiosildenafil in health supplements. J. Pharm. Biomed. Anal., 47, 279-284 (2008).
13) Reepmeyer, J. C., d'Avignon, D. A.: Structure elucidation of thioketone analogues of sildenafil detected as adulterants in herbal aphrodisiacs. J. Pharm. Biomed. Anal., 49, 145-150 (2009).
14) Li, L., Low, M. Y., Ge, X., Bloodworth, B. C., Koh, H. L.: Isolation and structural elucidation of dapoxetine as an adulterant in a health supplement used for sexual performance. J. Pharm. Biomed. Anal., 50, 724-728 (2009).
15) Low, M. Y., Li, L., Ge, X., Kee, C. L., Koh, H. L.: Isolation and structural elucidation of flibanserin as an adulterant in a health supplement used for female sexual performance enhancement. J. Pharm. Biomed. Anal., 57, 104-108 (2011).
16) McCarty, E. J., Dinsmore, W. W.: Dapoxetine: an evidence-based review of its effectiveness in treatment of premature ejaculation. Core Evidence, 7, 1-14 (2012).
17) Palacios, S.: Hypoactive sexual desire disorder and current pharmacotherapeutic options in women. Women's Health, 7, 95-107 (2011).
18) Analytical method for udenafil (provisional translation from Japanese original) (Pharmaceutical and Food Safety Bureau, Compliance and Narcotics Division, Notification No. 0822010, Aug. 22, 2007), The Ministry of Health, Labour and Welfare, Japan.
19) Saisho K., Goda Y., Analysis of ED treatment medicines and their analogues in capsule base for health foods using LC/MS and DART-TOFMS (provisional translation from Japanese original), In "Studies on surveillance, analysis and hazard assessment of illegal drugs", Annual report of Health and Labour Science Research Grant, 17-23 (2010).

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ORIGINAL PAPER

Chemical analysis reveals the botanical origin of shatavari products and confirms the absence of alkaloid asparagamine A in *Asparagus racemosus*

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Abstract Shatavari—a famous Ayurveda materia medica used mainly as a tonic for women—is distributed in health food products all over the world. The Ayurvedic Pharmacopoeia of India identifies the botanical origin of shatavari as the tuberous root of *Asparagus racemosus*. We recently investigated by DNA analysis the botanical origin of shatavari products on the Japanese market. The results suggested that their botanical origin was *Asparagus*; however, species identification was difficult. In this study, we analyzed steroidal saponins, including those specific to this plant, in these products and confirmed their origin as *A. racemosus*. Next, alkaloid analyses of an authentic *A. racemosus* plant and these products were performed, because several papers have reported the isolation of a pyrrolo[1,2-a]azepine alkaloid, asparagamine A, from this plant. Our results suggest that neither plant material nor products contained asparagamine A. It has been pointed out that *Stemona* plants are sometimes mistaken for shatavari, because their tuberous roots have a similar shape to that of *A. racemosus*, and pyrrolo[1,2-a]azepine alkaloids are thought to be *Stemona*-specific. These data strongly suggest that *A. racemosus* does not contain asparagamine A, and that previous isolation of asparagamine A from materials claimed as originating from *A. racemosus* was likely caused by misidentification of *Stemona* plants as *A. racemosus*.

Keywords Shatavari · *Asparagus racemosus* · *Stemona* plants · Asparagamine A

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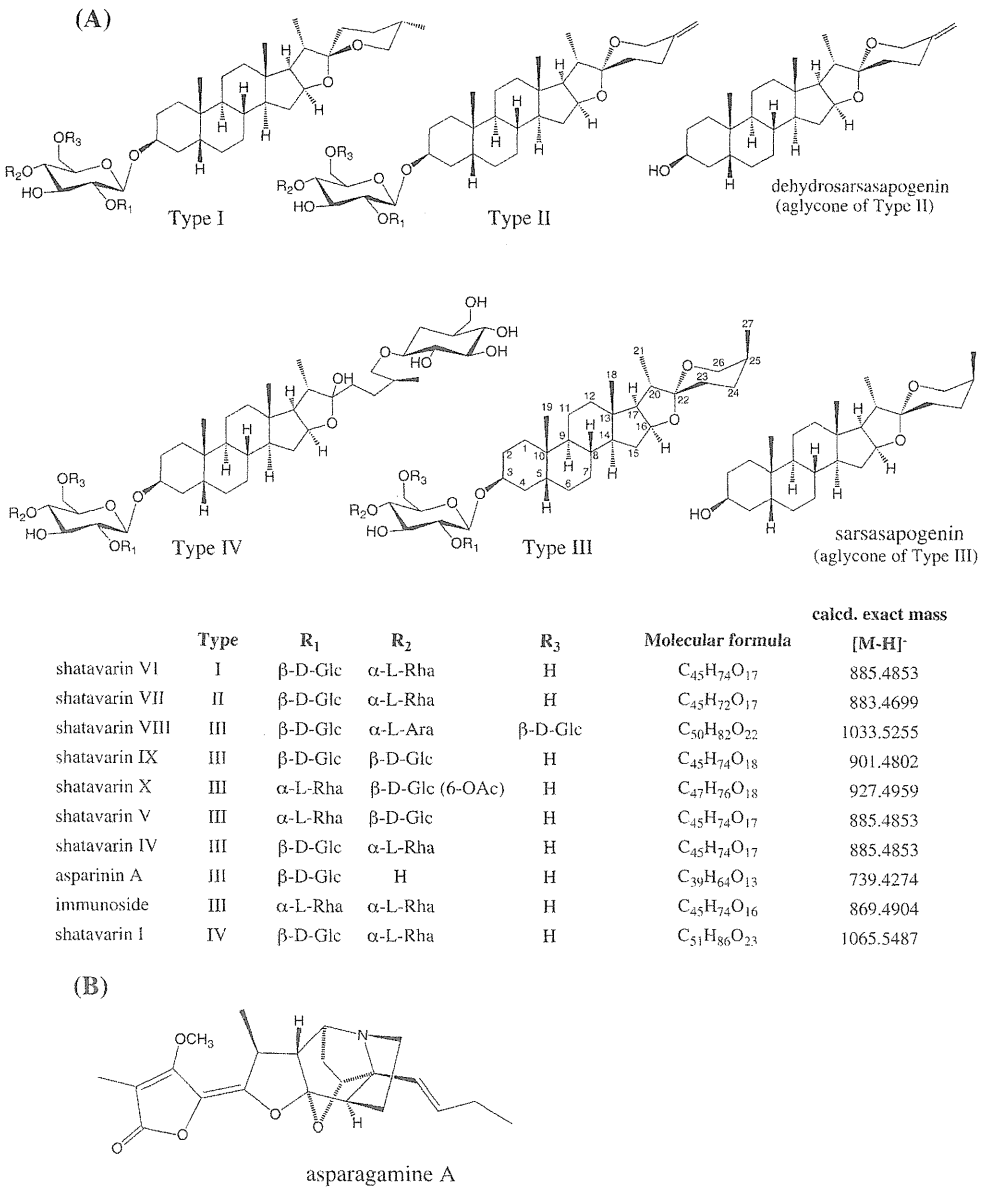
Introduction

Shatavari is a materia medica used in Ayurveda as a galactogogue, aphrodisiac, anodyne, and diuretic since time immemorial [1]. The Ayurvedic Pharmacopoeia of India specifies the botanical origin of shatavari as the tuberous root of *Asparagus racemosus* (Asparagaceae; Liliaceae) [2], which is found mainly in forests throughout India. Various pharmacological studies have manifested the further ability of *A. racemosus* extracts to modulate the immune system and its application in treating neurological disorders, diarrhea, and dyspepsia [3–7].

Steroidal saponins such as shatavarins (Fig. 1), isoflavones, and toxic alkaloid asparagamine A (Fig. 1) have been reported previously as constituents of *A. racemosus* roots [8–12]. However, pyrrolo[1,2-a]azepine alkaloids such as asparagamine A are also known to be distributed widely in *Stemona* plants (Stemonaceae) [13]. *Stemona* comprises about 25 species, most of which have tuberous roots. On local markets in China and Southeast Asia, the same vernacular names are used sometimes even for representatives of other plant families because of the similar shape of their roots. Under these circumstances, several reports have pointed out that *A. racemosus* could have been confused with *Stemona* plants, and that isolation of asparagamine A from *A. racemosus* could have resulted from the misidentification of *Stemona* plants as *A. racemosus* [13–15].

Given the increasing interest in alternative medicines, a worldwide market for shatavari, mainly as a tonic for women, has developed. Since *A. racemosus* seems to be confused with *Stemona* plants, there is a possibility that *Stemona* plants have been used as the source plants of these shatavari products. We recently investigated the botanical origin of 11 shatavari products on the Japanese market by DNA analysis. The results revealed that none of the



	Type	R_1	R_2	R_3	Molecular formula	calcd. exact mass [M-H]$^-$
shatavarin VI	I	β-D-Glc	α-L-Rha	H	$C_{45}H_{74}O_{17}$	885.4853
shatavarin VII	II	β-D-Glc	α-L-Rha	H	$C_{45}H_{72}O_{17}$	883.4699
shatavarin VIII	III	β-D-Glc	α-L-Ara	β-D-Glc	$C_{50}H_{82}O_{22}$	1033.5255
shatavarin IX	III	β-D-Glc	β-D-Glc	H	$C_{45}H_{74}O_{18}$	901.4802
shatavarin X	III	α-L-Rha	β-D-Glc (6-OAc)	H	$C_{47}H_{76}O_{18}$	927.4959
shatavarin V	III	α-L-Rha	β-D-Glc	H	$C_{45}H_{74}O_{17}$	885.4853
shatavarin IV	III	β-D-Glc	α-L-Rha	H	$C_{45}H_{74}O_{17}$	885.4853
asparinin A	III	β-D-Glc	H	H	$C_{39}H_{64}O_{13}$	739.4274
immunoside	III	α-L-Rha	α-L-Rha	H	$C_{45}H_{74}O_{16}$	869.4904
shatavarin I	IV	β-D-Glc	α-L-Rha	H	$C_{51}H_{86}O_{23}$	1065.5487

asparagamine A

Fig. 1 Chemical structures of **a** steroidal saponins and **b** asparagamine A reported previously from the roots of *Asparagus racemosus*

examined products contained *Stemona* plants and the botanical origin of all products was *Asparagus* [16]. However, species identification by DNA analysis alone was difficult because few or no nucleotide substitutions could be detected in the sequences of the regions frequently used in molecular phylogenetic studies among the 24 *Asparagus* species [17].

As chemotaxonomic studies, several *Asparagus* species have already been investigated and found to contain steroidal saponins [8, 18–20]. Among *Asparagus* species, *A. racemosus* has been reported to contain steroidal saponins that are specific to them, and have Type I or Type II structures, namely shatavarin VI and VII (Fig. 1) [8]. Therefore, we analyzed steroidal saponins in these shatavari products to confirm that these products undoubtedly originated from *A. racemosus* from the viewpoint of chemotaxonomy.

Moreover, we also performed alkaloid analyses of an authentic *A. racemosus* sample and these shatavari products to reveal whether shatavari contained asparagamine A or not.

Materials and methods

Plant materials and reagents

Details of the shatavari products used in this study are shown in Table 1. These products were purchased from Internet stores and are the same as those used in our previous study [16].

Authentic plants of *A. racemosus* and *S. collinsae* were kindly provided by A. Kanno of Tohoku University and

Table 1 Details of the commercial shatavari products used in this study

Sample no.	Product form	Composition[a]
Sha-1	Powder	100 % shatavari powder
Sha-2	Powder	100 % shatavari powder
Sha-3	Granule	Shatavari 0.56 g with sugar 4.44 g/5 g
Sha-4	Granule	Shatavari with sugar and water
Sha-5	Powder	Shatavari powder
Sha-6	Tablet	Shatavari churna 500 mg/tablet
Sha-7	Capsule	Shatavari 400 mg/capsule
Sha-8	Capsule	Root extract 500 mg/capsule
Sha-9	Capsule	Shatavari 250 mg/capsule
Sha-10	Capsule	Root extract 500 mg/capsule
Sha-11	Tablet	Root extract 250 mg, stem and root powder 400 mg/tablet

[a] Each composition is according to the supplier's description of the product

S. Jiwajinda of Kasetsart University, Thailand, respectively. Voucher specimens (ARC001, *A. racemosus*; Sc-3, *S. collinsae*) were deposited at the National Institute of Health Sciences (NIHS), Japan.

An authentic standard of sarsasapogenin used in LC–MS analysis was purchased from ChromaDex (Irvine, CA). Acetonitrile was of HPLC grade and Milli Q water was used; other reagents were of guaranteed grade.

Steroidal saponin analysis

Sample preparation

Methanol (5 mL) was added to 50 mg Sha-8 and Sha-11 (derived from shatavari extracts) and 500 mg of the other sample products (except Sha-3 and Sha-4), and shaken for extraction (300 min^{-1}, 15 min). After extraction, each extract was centrifuged at 1,200×*g* for 5 min, and 1 μL of the supernatant was used for LC–MS analysis. For Sha-3 and Sha-4 samples, 20 mL methanol was added to 4.46 g of each sample (corresponding to 500 mg shatavari on the basis of the product information of Sha-3), and shaken for extraction (300 min^{-1}, 15 min), and the residues were filtered out. After removal of the solvent from each filtrate under a vacuum, the extract was dissolved with 5 mL methanol and 1 μL of the resultant mixture was used for LC–MS analysis.

LC–TOFMS analysis

LC–TOFMS analyses were performed using an LCMS-IT-TOF (Shimadzu, Kyoto, Japan) equipped with an XBridge C18 (2.1 × 100 mm, 3.5 μm; Waters, Milford, MA). The mobile phase consisted of 0.3 % acetic acid as solvent A and acetonitrile containing 0.3 % acetic acid as solvent B. Analyses were performed at a flow rate of 0.4 mL/min using the following gradient program: 10 % B (0.00 min)–77.5 % B (15 min). The column oven temperature was 40 °C, and the measurement wavelength of the PDA detector was 190–800 nm. MS data were obtained in the electrospray ionization (ESI)-negative mode using the following parameters: capillary voltage 1,570 V, dry gas (N_2) 100 kPa, nebulizer gas 1.5 mL/min, and heat block temperature 200 °C. The exact mass of each peak was corrected using glycyrrhizin as an external standard (*m/z* 821.3965).

LC–QMS analysis

LC–QMS analyses were performed using an LCMS-2020 (Shimadzu), and the column, mobile phase, and gradient program were the same as that of LC–TOFMS analysis. MS data were obtained in the ESI-negative mode with selected ion monitoring (SIM) (*m/z* 739, 869, 883, 885, 901, 927, 1033, 1065) using the following parameters: capillary voltage 1100 V, dry gas (N_2) 10 L/min, and dry gas temperature 250 °C. Nebulizer gas and heat block temperatures were as described above.

LC–MS/MS analysis

LC–MS/MS analyses were performed using the UFLC (Shimadzu)—LTQ Orbitrap XL (ThermoFisher Scientific, Waltham, MA) equipped with Hypersil-GOLD (2.1 × 100 mm, 3.5 μm; ThermoFisher Scientific). Analyses were performed at a flow rate of 0.3 mL/min using the following gradient program: 10 % B (0.00 min)–70 % B (15 min). MS data were obtained in the ESI-positive mode using the following parameters: ESI needle voltage 3,500 V, capillary temperature 300 °C.

Sapogenin analysis

Acid hydrolysis

A methanol extract of each shatavari product (800 μL) prepared in saponin analysis was hydrolyzed with 400 μL 6 *N* HCl for 3 h at 80 °C. The reaction mixture was then centrifuged at 15,500×*g* for 1 min, and 1 μL supernatant was used for LC–MS analysis.

LC–TOFMS analysis

LC–TOFMS analyses for sapogenin were performed as were those for steroidal saponin, except that the mobile phase was water:acetonitrile = 15:85 in an isocratic

condition, and that MS data were obtained in the ESI-positive mode.

Alkaloid analysis

Isolation of asparagamine A

Authentic plant samples of *S. collinsae* (1.75 g) was subjected to overnight extraction with methanol (20 mL × 2). The methanol extract (1.03 g) was partitioned under acid and basic conditions with ethyl acetate (20 mL × 2), and 8.2 mg of the basic fraction was obtained. Asparagamine A (1.5 mg) was isolated by preparative HPLC of this fraction. The column and mobile phase were Inertsil PREP-ODS (20 × 250 mm; GL Sciences, Tokyo, Japan) and 0.3 % acetic acid:acetonitrile = 85:15, respectively. The structure was confirmed by comparing NMR (ECA-500; Jeol, Tokyo, Japan) data and MS (LCMS-IT-TOF; Shimadzu) data with those in the literature [21].

LC–TOFMS analysis

Methanol extracts of shatavari products were analyzed by LC–TOFMS to confirm the presence of asparagamine A in the shatavari products. LC–TOFMS analyses for alkaloids were performed as were those for steroidal saponin, except that the gradient program was 10 % B (0.00 min)–30 % B (15 min), and that MS data were obtained in the ESI-positive mode.

Results and discussion

Analyses of steroidal saponins in shatavari products

A. racemosus has been reported to contain ten steroidal saponins, including those which are specific to *A. racemosus* [8]. LC–MS analysis of steroidal saponins in the shatavari products used in this study was performed to reveal that *A. racemosus* was the source plant of these products.

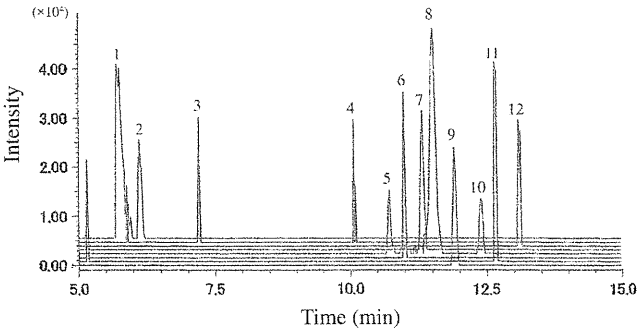
First, we analyzed the Sha-2 sample simply because we had the largest amount of this product. Each component was estimated by its exact mass because authentic samples of ten steroidal saponins could not be obtained. Negative-mode LC–TOFMS analysis of the methanol extract of Sha-2 resulted in 14 peaks, which reflected eight $[M–H]^-$ ions at *m/z* 739, 869, 883, 885, 901, 927, 1033, and 1065, corresponding to the same molecular formula as those of the ten steroidal saponins. Each exact mass of 12 peaks out of the 14 showed a certain value within ±3 mmu from those of the corresponding components, except the peak at *m/z* 927 corresponding to shatavarin X, which showed a value within +8 mmu (Fig. 2). The peak detected at a retention time of 11.5 min (peak no. 8) was estimated to be shatavarin IV, owing to its mass (*m/z* 885.4861) and the elution order of the ten saponins under the same mobile phase [8]. The peak ascribed to shatavarin IV was also the biggest also in the LC–MS analysis in SIM mode. This result agrees with the report that shatavarin IV is the main component of steroidal saponins in *A. racemosus* [8].

The peak detected at a retention time of 11.0 min (peak no. 6) was estimated to be shatavarin VII, which is a saponin specific to *A. racemosus*. In the positive mode, MS/MS analysis of this peak showed the fragment ion (*m/z* = 415) corresponding to its aglycone, dehydrosarsasapogenin (Fig. 1).

Next, acid-hydrolyzed methanol extracts of Sha-2 were analyzed by LC–TOFMS in positive mode, resulting in one peak showing a retention time (t_R = 3.9 min) and mass number (*m/z* = 417.30) consistent with those of authentic sarsasapogenin (Fig. 1), which is the main aglycone of shatavarins (Fig. 3). Combined with the data of the hydrolyzed aglycone, the above MS analyses of Sha-2 methanol extract indicated that the extract contained steroidal saponins reported from *A. racemosus*.

In a similar way, the methanol extracts and their acid-hydrolysates of the other shatavari products were analyzed by LC–MS, and all the data showed the presence of the peaks ascribed to shatavarins or those corresponding to sarsasapogenin.

Of ten steroidal saponins, those containing a sarsasapogenin aglycone (Fig. 1, Type III) are found commonly in the Asparagaceae family. Shatavarin I and IV (Type IV)



Peak No.	Time	*m/z*	estimated compound	Peak No.	Time	*m/z*	estimated compound
1	5.8	1065.5493	Shatavarin I*	7	11.3	901.4819	Shatavarin IX
2	6.1	1065.5498	Shatavarin I*	8	11.5	885.4861	Shatavarin IV
3	7.2	1033.5235	Shatavarin III**	9	11.9	739.4251	asparinin A
4	10.1	1033.5242	Shatavarin III**	10	12.4	885.4851	Shatavarin V
5	10.7	885.4853	Shatavarin VI	11	12.7	869.4889	immunoside
6	11.0	883.4711	Shatavarin VII	12	13.1	927.5040	Shatavarin X

*Both peaks are possible to represent Shatavarin I
**Both peaks are possible to represent Shatavarin III

Fig. 2 Mass spectrometry (MS) chromatogram of the methanol extract from the Sha-2 product on LC–MS analysis for saponins

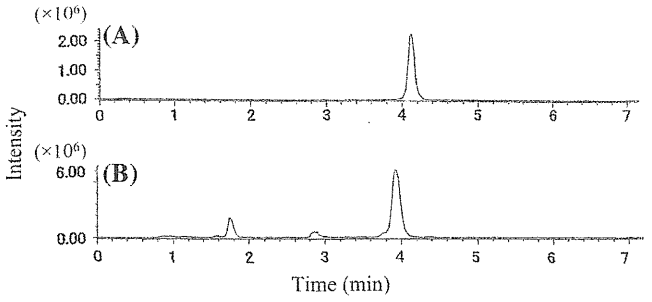


Fig. 3 MS chromatograms of **a** sarsasapogenin and **b** the acid-hydrolysate of methanol extract from Sha-2 product on LC–MS analysis (m/z = 417.3)

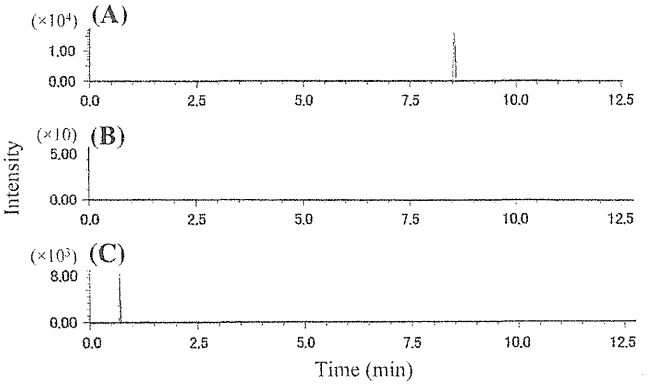


Fig. 4 MS chromatograms of **a** asparagamine A (0.1 ng), and the methanol extract from **b** an *A. racemosus* plant and **c** the Sha-2 product on LC–MS analysis (m/z = 386.1–386.2)

have been isolated previously from different species of *Asparagus* [18, 19]. However, the saponins that possess rare aglycones, such as with the 22-S configuration in the F ring of sarsasapogenin (Type I) or dehydrosarsasapogenin (Type II), have never been found in Asparagaceae family plants other than *A. racemosus*, although these types of saponins have been found in two species of *Dichelostemma multiflorum* [22] and *Yucca schidigera* [23] in the Liliaceae family and one species of *Helleborus macranthus* [24] in the Ranunculaceae family.

LC–MS analyses of all shatavari products showed the representative ten steroidal saponins, including two that are specific to *A. racemosus* (Shatavarins VI and VII), and the biggest peak was estimated as shatavarin IV, which is reported as the predominant steroidal saponins in *A. racemosus*. Considered together with the results of our previous DNA analysis [16], our LC–MS results strongly suggest that the source plant of all products was *A. racemosus*.

Analyses of alkaloid asparagamine A in *A. racemosus* authentic plant and shatavari products

In order to investigate whether shatavari contains asparagamine A or not, authentic *A. racemosus*, which was unequivocally identified by morphologic and genetic analysis, and shatavari products whose botanical origin was confirmed to be *A. racemosus* were subsequently subjected to LC–MS analysis.

First, 1.5 mg asparagamine A was prepared from *S. collinsae* from which the isolation of this compound has been reported [14, 21, 25]. The LC–MS analyses of this authentic sample revealed that the detection limit of asparagamine A was 0.1 ng (Fig. 4a).

The methanol extract of *A. racemosus* roots was analyzed under the same conditions, and no peak corresponding to asparagamine A was detected (Fig. 4b). Also, none of the shatavari product extracts showed a peak corresponding to asparagamine A (Fig. 4c).

We next investigated most of the mass peaks from all of the methanol extracts. More than 130 pyrrolo[1,2-a]azepine alkaloids, of which the molecular weight is within the range of 223–563, are known [26, 27], and no peaks of $[M+H]^+$ corresponding to these alkaloids appeared. Considering the biosynthetic pathway of asparagamine A, these data also support our result that asparagamine A could not be detected in *A. racemosus* samples.

While we could not detect asparagamine A from authentic *A. racemosus* and its products used in this study, three papers have reported the isolation of asparagamine A from *A. racemosus* [10–12]. However, two of the three papers did not describe how the species of plant materials used were identified, and the other described that identification of the plant material used was performed only by the shape of its roots. Furthermore, the dihydrophenanthrene derivative, racemosol (=stemanthrene D), which has been shown to be a specific stilbenoid in *Stemona* species such as *S. collinsae* [28, 29], was also isolated with asparagamine A from a questionable *A. racemosus* sample [30]. Considering our results and the fact that the roots of *A. racemosus* and *Stemona* plants have similar shapes, as mentioned above, isolation of asparagamine A from *A. racemosus* [10–12] is thought to have been caused by misidentification of *Stemona* plants as *A. racemosus*.

It is of interest whether shatavari contains alkaloids or not from the viewpoint of chemotaxonomy. In addition, this chemotaxonomic study provides important information for classifying shatavari products as foods in Japan, because the presence of alkaloids is one of the criteria for distinguishing pharmaceuticals from non-pharmaceuticals. Botanical origin of used plant material is one of the most important things for natural product chemistry. However, sometimes it is difficult to morphologically identify it, when the material is purchased in markets or collected by unprofessional persons. In these cases, combination study

of chemotaxonomic and genetic approaches, as shown in our reports, is considered as the most powerful tool for proper identification of origin.

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References

1. Bopana S, Saxena S (2007) *Asparagus racemosus*—ethnopharmacological evaluation and conservation needs. J Ethnopharmacol 110:1–15
2. The Ayurvedic Pharmacopoeia of India (2008) 1st edn, part I, vol IV. Ministry of Health and Family Welfare, India
3. Gautam M, Diwanay S, Gairola S, Shinde Y, Patki P, Patwardhan B (2004) Immunoadjuvant potential of *Asparagus racemosus* aqueous extract in experimental system. J Ethnopharmacol 91:251–255
4. Bhattacharya A, Murugandam AV, Kumar V, Bhattacharya SK (2002) Effect of polyherbal formulation, EuMil, on neurochemical perturbations induced by chronic stress. Indian J Exp Biol 40:1161–1163
5. Parihar MS, Hemnani T (2004) Experimental excitotoxicity provokes oxidative damage in mice brain and attenuation by extract of *Asparagus racemosus*. J Neural Transm 111:1–12
6. Venkatesan N, Thiyagarajan V, Narayanan S, Arul A, Raja S, Kumar SGV, Rajarajan T, Perianayagam JB (2005) Anti-diarrhoeal potential of *Asparagus racemosus* wild root extracts in laboratory animals. J Pharm Pharm Sci 8:39–45
7. Dalvi SS, Nadkarni PM, Gupta KC (1990) Effect of *Asparagus racemosus* (Shatavari) on gastric emptying time in normal healthy volunteers. J Postgrad Med 36:91–94
8. Hayes PY, Jahidin AH, Lehmann R, Penman K, Kitching W, De Voss JJ (2008) Steroidal saponins from the roots of *Asparagus racemosus*. Phytochemistry 69:796–804
9. Saxena VK, Chourasia S (2001) A new isoflavone from the roots of *Asparagus racemosus*. Fitoterapia 72:307–309
10. Sekine T, Fukasawa N, Kashiwagi Y, Ruangrungsi N, Murakoshi I (1994) Structure of asparagamine A, a novel alkaloid from *Asparagus racemosus*. Chem Pharm Bull 42:1360–1362
11. Sekine T, Ikegami F, Fukusawa N, Kashiwagi Y, Aizawa T, Fujii Y, Ruangringsi N, Murakoshi I (1995) Structure and relative stereochemistry of a new polycyclic alkaroid, asparagamine A, showing anti-oxytocin activity, isolated from *Asparagus racemosus*. J Chem Soc Perkin Trans 1:391–393
12. Wiboonpun N, Phuwapraisirisan P, Tip-pyang S (2004) Identification of antioxidant compound from *Asparagus racemosus*. Phytother Res 18:771–773
13. Gregner H (2006) Structural relationships, distribution and biological activities of *Stemona* alkaloids. Planta Med 72:99–113
14. Brem B, Seger C, Pacher T, Hofer O, Vajrodaya S, Greger H (2002) Feeding deterrence and contact toxicity of *Stemona* alkaloids—a source of potent natural insecticides. J Agric Food Chem 50:6383–6388
15. Brüggemann M, McDonald AI, Overman LE, Rosen MD, Schwink L, Scott JP (2003) Total synthesis of (±)-didehydrostemofoline (asparagamine A) and (±)-isodidehydrostemofoline. J Am Chem Soc 125:15284–15285
16. Kumeta Y, Maruyama T, Wakana D, Kamakura H, Goda Y (2011) Method for identifying the botanical origin of shatavari products and its application for survey analysis of products in the Japanese market. Jpn J Food Chem Safety 18:163–167
17. Fukuda T, Ashizawa H, Suzuki R, Ochiai T, Nakamura T, Kanno A, Kameya T, Yokoyama J (2005) Molecular phylogeny of the genus *Asparagus* (Asparagaceae) inferred from plastid *petB* intron and *petD-rpoA* intergenic spacer sequences. Plant Species Biol 20:121–132
18. Zhang HJ, Sydara K, Tan GT, Ma C, Southavong B, Soejarto DD, Pezzuto JM, Fong Harry HS (2004) Bioactive constituents from *Asparagus cochinchinensis*. J Nat Prod 67:194–200
19. Huang X, Kong L (2006) Steroidal saponins from roots of *Asparagus officinalis*. Steroids 71:171–176
20. Sautour M, Miyamoto T, Lacaille-Dubois MA (2007) Steroidal saponins from *Asparagus acutifolius*. Phytochemistry 68:2554–2562
21. Jiwajinda S, Hirai N, Watanabe K, Santisopasri V, Chuengsamarnyart N, Koshimizu K, Ohigashi H (2001) Occurrence of the insecticidal 16,17-didehydro-16(*E*)-stemofoline in *Stemona collinsae*. Phytochemistry 56:693–695
22. Inoue T, Mimaki Y, Sashida Y, Nikaido T, Ohmoto T (1995) Steroidal saponins from *Dichelostemma multiflorum* tubers and their inhibitory activity on cAMP phosphodiesterase. Phytochemsitry 39:1103–1110
23. Miyakoshi M, Tamura Y, Masuda H, Mizutani K, Tanaka O, Ikeda T, Ohtani K, Kasai R, Yamasaki K (2000) Antiyeast steroidal saponins from *Yucca schidigera* (Mohave Yucca), a new antifood-deteriorating agent. J Nat Prod 63:332–338
24. Petricic J, Tarle D, Kupinic M, Tarle D (1996) Structure of macranthogenin, the main aglycon of saponin in subsurface parts of *Helleborus macranthus*. Acta Pharm Jugoslavica 19:149–154
25. Seger C, Mereiter K, Kaltenegger E, Pacher T, Greger H, Hofer O (2004) Two pyrrolo[1,2-a]azepine type alkaloids from *Stemona collinsae* Craib: structure elucidations, relationship to asparagamine A, and a new biogenetic concept of their formation. Chem Biodivers 1:265–279
26. Pilli RA, Ferreira de Oliveira MC (2000) Recent progress in the chemistry of the *Stemona* alkaloids. Nat Prod Rep 17:117–127
27. Pilli RA, Rosso GB, Ferreira de Oliveira MC (2010) The chemistry of Stemona alkaloids: an update. Nat Prod Rep 27:1908–1937
28. Pacher T, Seger C, Engelmeier D, Vajrodaya S, Hofer O, Greger H (2002) Antifungal stilbenoids from *Stemona collinsae*. J Nat Prod 65:820–827
29. Kostecki K, Engelmeier D, Pacher T, Hofer O, Vajrodaya S, Greger H (2004) Dihydrophenanthrenes and other antifungal stilbenoids from *Stemona* cf. *pierrei*. Phytochemistry 65:99–106
30. Sekine T, Fukusawa N, Murakoshi I, Ruangrungsi N (1997) A 9,10-dihydrophenanthrene from *Asparagus racemosus*. Phytochemistry 44:763–764

Two New Pyrrolidine Alkaloids, Codonopsinol C and Codonopiloside A, Isolated from *Codonopsis pilosula*

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A new pyrrolidine alkaloid codonopsinol C (1), and pyrrolidine alkaloidal glycoside, codonopiloside A (2), were isolated from the roots of *Codonopsis pilosula*, along with four known pyrrolidine alkaloids, codonopsinol A (3), codonopsinol B (4), codonopyrrolidium B (5), and radicamine A (6). The structures of the new compounds were established by acid hydrolysis and spectroscopic methods. We describe those structures in this paper.

Key words *Codonopsis pilosula*; pyrrolidine alkaloid; Japanese Pharmacopoeia; NMR; Campanulaceae

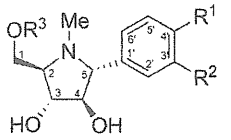
The crude drug *Tojin* consists of the roots of *Codonopsis pilosula* (Franch.) Nannf. and *C. tangshen* Oliv. In Japan, *Tojin* has been used as a component of Kampo foumulae such as "Umpito" and as an ingredient in several over the counter (OTC) crude drug products. It is categorized as a raw material used exclusively as in pharmaceuticals. Since the crude drug is recognized to be an essential raw material for public health and medical treatment in Japan, the Japanese Pharmacopoeia (JP) committee is discussing its standardization for the purpose of adopting into JP.

In our previous paper, we started to investigate the chemical constituents of crude drugs purchased in Japan to obtain information about secondary metabolites. We isolated three new triterpenyl esters, codonopilates A–C.[1] In order to discuss its standardization and regulation, alkaloidal components are very important. In 1988, Wang *et al.* and Liu isolated pyridine alkaloid, nicotinic acid and 5-hydroxy-2-pyridinemethanol,[2] and β-carboline alkaloid perlolyrine[3] from *C. pilosula*, respectively. Tsai and Lin identified pyrrolidine alkaloids codonopyrrolidiums A and B (**5**)[4] from *C. tangshen*. Ishida *et al.* reported codonopsinol (**7**) isolated from *C. clematidea*.[5] The absolute stereochemistry of **7** was revised by comparison to the optical rotation of synthetic compound.[6,7] Li *et al.* isolated quaternary ammonium alkaloids codotubulosines A and B[8] from *C. tubulosa*. In this paper, we deal with the isolation and structural elucidation of the new pyrrolidine alkaloids, codonopsinol C (**1**) and codonopiloside A (**2**).

Results and Discussion

The alkaline BuOH extract of Codonopsis Radix was purified by various chromatographic techniques to give two new pyrrolidine alkaloids codonopsinol C (**1**) and codonopiloside A (**2**), along with four known compounds, (2*R*,3*R*,4*R*,5*R*)-2-(3-hydroxy-4-methoxyphenyl)-5-(hydroxymethyl)-1-methylpyrrolidine-3,4-diol (**3**)[6] (2*R*,3*R*,4*R*,5*R*)-2-(hydroxymethyl)-5-(4-methoxyphenyl)-1-methylpyrrolidine-3,4-diol (**4**),[6] codonopyrrolidium B (**5**),[4] and radicamine A (**6**)[9] (Fig. 1). Compounds **3** and **4** were new compounds as natural products, therefore we named **3** and **4** as codonopsinol A and B, respectively.

The molecular formula of codonopsinol C (**1**) was confirmed as $C_{12}H_{17}NO_4$ by direct analysis in real-time-time-of-flight-mass spectra (DART-TOF-MS). The ^{1}H-NMR spectrum of **1** showed 1,4-substituted phenyl protons [δ 6.75d (8.5, 2H), δ 7.17d (8.5, 2H)], an *N*-methyl proton (δ 2.16s, 3H), four methine protons (δ 3.04, δ 3.61, δ 3.92, δ 3.99) and oxygenated methylene protons [δ 3.80dd (4.3, 11.6), δ 3.84dd (4.3, 11.6)]. This result was very similar to that of **4**, except for the *O*-methyl group. Therefore, **3** was suggested to be the *O*-demethyl compound of **4**. The planar structure was elucidated by two dimensional (2D)-NMR spectra (Fig. 2). The detailed analysis of the ^{1}H–^{1}H correlation spectroscopy (COSY) spec-



codonopsinol C (**1**): R^1 = OH, R^2 = H, R^3 = H
codonopiloside A (**2**): R^1 = OMe, R^2 = H, R^3 = glucopyranosyl
codonopsinol A (**3**): R^1 = OMe, R^2 = OH, R^3 = H
codonopsinol B (**4**): R^1 = OMe, R^2 = H, R^3 = H
codonopsinol (**7**): R^1 = OMe, R^2 = OMe, R^3 = H

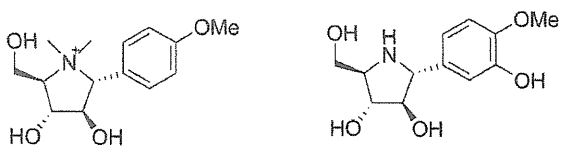


Fig. 1. Structures of Pyrrolidine Alkaloids Isolated from *C. pilosula*

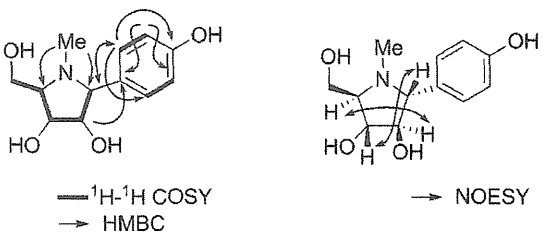


Fig. 2. ^{1}H–^{1}H COSY, HMBC and NOESY Spectra of **1**

The authors declare no conflict of interest.

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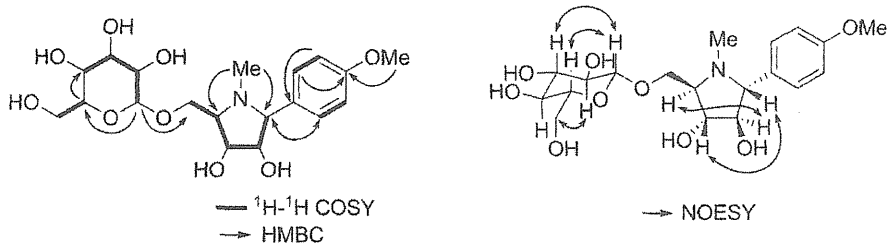

Fig. 3. ^{1}H–^{1}H COSY, HMBC and NOESY Spectra of **2**

trum showed correlations between 1-H_2 [δ 3.80dd (4.3, 11.6), δ 3.84dd (4.3, 11.6)] and 2-H [δ 3.04td (4.3, 4.8)], 2-H and 3-H [δ 3.99t (4.8)], 3-H and 4-H [δ 3.92dd (4.8, 6.5)], 4-H and 5-H [δ 3.61d (6.5)], and the analysis of heteronuclear multiple bond correlation (HMBC) spectra shown from *N*-methyl proton (δ 2.16s) to C-2 (δ 71.0) and C-5 (δ 75.3). These data suggested the presence of *N*-methyl-2-hydroxymethyl-pyrrolidine-3,4-diol moiety. Also, the HMBC correlations from 4-H to C-1′ (δ 132.2) and from 5-H to C-2′/C-6′ (δ 130.7) suggested that the phenyl moiety was connected to the C-5 of the pyrrolidine moiety. The relative stereochemistry was confirmed by the NOESY spectrum that showed 2-H to 4-H and 3-H to 5-H. The absolute stereochemistry was considered to be (2*R*, 3*R*, 4*R*, 5*R*), the same as in **4**, because of the comparison of the CD spectra of **4**[8] with that of **1**.

Codonopiloside A (**2**) showed the molecular formula as $C_{19}H_{29}NO_9$ by DART-TOF-MS. The ^{1}H-NMR spectrum of **2** showed 1,4-substituted phenyl protons [δ 6.90d (8.6, 2H), δ 7.28d (8.6, 2H)], a methoxy proton (δ 3.78s, 3H), an *N*-methyl proton (δ 2.16s, 3H), eight methine protons (δ 3.21, δ 3.24, δ 3.29, δ 3.29, δ 3.37, δ 3.69, δ 3.93, δ 4.11), an anomeric proton (δ 4.28) and two methylene protons [δ 3.73dd (4.0, 10.6), δ 4.30dd (4.0, 10.6), δ 3.67dd (4.0, 11.7), δ 3.88bd (11.7)]. Therefore, the structure of **2** suggested the glycosidal compound of **4**. The position of the *N*-methyl group was determined by the HMBC correlations of **2** from the *N*-methyl proton to C-2 (δ 69.6) and C-5 (δ 75.6). Therefore, the aglycone moiety of **2** was determined to be same as that of **4**. To determine the sugar moiety of **2**, it was hydrolyzed by 4 M HCl. In the results, (2*R*,3*R*,4*R*,5*R*)-2-(hydroxymethyl)-5-(4-methoxyphenyl)-pyrrolidine-3,4-diol[6] and D-glucose were afforded. The sugar moiety was identified as D-glucose by the analysis of ^{1}H-NMR spectrum of sugar part in **2** and the comparison of the optical rotation to standard compound (sugar moiety of **2**: $[\alpha]_D$ +49.8°, standard compound: $[\alpha]_D$ +50.7°). The anomeric proton of **2** was confirmed to be β-anomer as a result of the coupling constants of anomeric proton [δ 4.28d (8.0)]. The HMBC correlation from 1″-H to C-1 (δ 69.1) suggested that D-glucose was attached to C-1 of the pyrrolidine moiety. Therefore, **2** was confirmed to be codonopsinol C-1-*O*-β-D-glucopyranosyl and was named Codonopiloside A.

Conclusion

In our search of alkaloid compounds from *C. pilosula*, we found two new pyrrolidine alkaloids, codonopsinol C (**1**) and codonopiloside A (**2**), together with four known pyrrolidine alkaloid codonopsinols A (**3**) and B (**4**), codonopyrrolidium B (**5**) and radicamine A (**6**). Compounds **3** and **4** were already reported for the synthesized compound as (2*R*,3*R*,4*R*,5*R*)-2-(3-hydroxy-4-methoxyphenyl)-5-(hydroxymethyl)-1-methylpyrrolidine-3,4-diol and (2*R*,3*R*,4*R*,5*R*)-2-(hydroxymethyl)-5-(4-methoxyphenyl)-1-methylpyrrolidine-3,4-diol, respectively, but they were first isolated as natural compounds. Codonopiloside A (**1**), the glucosidal compound of this type of pyrrolidine alkaloids, was first reported in this paper.

Discussion

The pyrrolidine alkaloids isolated from *C. pilosula*, codonopsinol A (**3**), codonopsinol B (**3**), and radicamine A (**6**), were already reported to be α-glucosidase inhibitory.[8,9] In addition, pyrrolidine alkaloids, broussonetinines E and F isolated from the bark of *Broussonetia kazinoki*, were also α-glucosidase inhibitors.[10] Otherwise, some pyrrolidine alkaloids showed toxicity. For example, bgugaine is a hepatotoxin in rat and human liver cells.[11] Therefore, we think that the crude drug *Tojin*, which detects pyrrolidine alkaloids, should be used carefully as a raw material used exclusively as a pharmaceuticals, and its standardization by JP is very important for public health.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO (Tokyo, Japan) DIP-370 digital polarimeter, UV spectra on a Shimadzu (Kyoto, Japan) UV-2550 spectrophotometer, IR spectra on a JASCO FT-IR-5300 spectrophotometer and circular dichroism (CD) spectra on a JASCO J-720 spectropolarimeter. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL ECA-500 spectrometer (500.16 MHz for ^{1}H, 125.77 MHz for ^{13}C) and/or ECA-800 (800.14 MHz for ^{1}H, 201.20 MHz for ^{13}C) spectrometer, using tetramethylsilane as an internal standard. Coupling patterns are indicated as follows: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, and br=broad. Mass spectra were obtained on a JEOL DART ion source coupled to a AccuTOF JMS-100LC mass spectrometer. Preparative LPLC was performed on an EYELA ceramic pump VSP-3050, using a prepacked glass column, Yamazen SI-40B (26 mm i.d.×300 mm) or ODS-S-40B (26 mm i.d.×300 mm). HPLC was performed on a Shimadzu LC-8A pump with a flow rate of 4 mL/min using a GL Sciences (Tokyo, Japan). Inertsil SIL 100A, NH_2 (10 mm i.d.×250 mm), and/or a Kanto Chemical (Tokyo, Japan) Mightysil RP-18GP (10 mm i.d.×250 mm) prepacked column equipped with a Shimamura RI monitor YRD-883 or a Shimadzu SPD-6AV monitor.

Plant Material *Codonopsis pilosula* (FRANCH.) NANNF. was purchased from Uchida Wakanyaku (Tokyo, Japan). A voucher specimen was deposited at the National Institute of Health Sciences, Japan.

Extraction and Isolation The dried roots of *C. pilosula* (500 g) were extracted with MeOH (2×2 L) at room tempera-

Table 1. ^{1}H- and ^{13}C-NMR Data of **1** and **2** in Methanol-d_4

No.	Codonopsinol C (**1**)		Codonopiloside A (**2**)	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	60.8	3.80 dd (4.3, 11.6)	69.1	3.73 dd (4.0, 10.6)
		3.84 dd (4.3, 11.6)		4.30 dd (4.0, 10.6)
2	71.0	3.04 td (4.3, 4.8)	69.6	3.21 m
3	79.9	3.99 t (4.8)	80.4	4.11 t (4.6)
4	85.5	3.92 dd (4.8, 6.5)	86.1	3.93 dd (4.6, 6.9)
5	75.3	3.61 d (6.5)	75.6	3.69 d (6.9)
1′	132.2	—	133.3	—
2′/6′	130.7	7.17 d (8.5)	130.6	7.28 d (8.6)
3′/5′	116.1	6.75 d (8.5)	114.8	6.90 d (8.6)
4′	158.1	—	160.8	—
N-CH_3	35.0	2.16 s	35.0	2.16 s
O-CH_3			55.7	3.78 s
1″			105.1	4.28 d (8.0)
2″			75.3	3.24 dd (8.0, 9.1)
3″			77.9	3.37 t (9.1)
4″			71.7	3.29 m
5″			78.1	3.29 m
6″			62.8	3.67 dd (4.0, 11.7)
				3.88 brd (11.7)

ture, and the solvent was evaporated *in vacuo*. The residue was suspended in H_2O and extracted with $CHCl_3$. The H_2O layer was adjusted for pH 2 with 4 M HCl, and then extracted with *n*-BuOH. The residual aqueous layer was adjusted for pH 11 with 25% NH_4OH and then extracted with *n*-BuOH. The alkaline BuOH layer (12.0 g) was chromatographed on Sephadex LH-20 [$CHCl_3$–MeOH (1 : 1)] to give six fractions. Fraction 2 was purified by HPLC with 80% CH_3CN to give lobetyolinin (25 mg) and codonopiloside A (**2**: 4 mg). The third fraction was separated by HPLC with 80% CH_3CN to give codonopyrrolidium B (**5**: 15 mg), codonopsinol B (**3**: 3 mg), and codonopsinol A (**3**: 3 mg). The fifth fraction was repeatedly purified by HPLC with 80% CH_3CN and EtOH to give radicamine A (**6**: 11 mg) and codonopsinol C (**1**: 3 mg).

Codonopsinol C (**1**): Colorless amorphous gum: $[\alpha]_D$ −9° (c=0.60, MeOH). IR (ATR) ν_{max} cm^{-1}: 3333 (–OH), 1602, 1512 (phenyl). UV (MeOH) λ_{max}nm (logε): 277 (3.09), 226 (3.86). CD (MeOH) $\Delta\varepsilon$ (nm): +3.88 (278), +5.02 (225). The ^{1}H- and ^{13}C-NMR spectral data are summarized in Table 1. DART-TOF-MS m/z 240.1244 $[M+H]^+$ (Calcd for $C_{12}H_{18}NO_4$: 240.1236).

Codonopiloside A (**2**): Colorless amorphous gum: $[\alpha]_D$ −15° (c=0.57, MeOH). IR (ATR) ν_{max} cm^{-1}: 3354 (–OH), 1508 (phenyl). UV (MeOH) λ_{max}nm (logε): 275 (3.05), 226 (3.81). CD (MeOH) $\Delta\varepsilon$ (nm): +3.61 (275), −3.12 (227), +4.21 (214). The ^{1}H- and ^{13}C-NMR spectral data are summarized in Table 1. DART-TOF-MS m/z 416.1913 $[M+H]^+$ (Calcd for $C_{19}H_{30}NO_9$: 416.1920).

The Hydrolysis of 2 Compound **2** (2 mg) was dissolved in 4 M HCl (10 mL), and the solution was refluxed for 3 h. After cooling, the reaction mixture was extracted with AcOEt, and then the aqueous layer was adjusted for pH 11 with 25% NH_4OH, and extracted with AcOEt. The alkaline organic layer was concentrated *in vacuo* to give (2*R*,3*R*,4*R*,5*R*)-2-(hydroxymethyl)-5-(4-methoxyphenyl)-pyrrolidine-3,4-diol (0.5 mg). The residue was purified by HPLC on an amino column with 75%CH_3CN to give D-glucose (0.3 mg). (2*R*,3*R*,4*R*,5*R*)-2-(Hydroxymethyl)-5-(4-methoxyphenyl)-pyrrolidine-3,4-diol was confirmed by comparison to data in the literature[6] and D-glucose was decided by comparison with the standard compound.

Codonopsinol A (**3**): Colorless amorphous gum: $[\alpha]_D$ +5° (c=0.30, MeOH). IR (ATR) ν_{max} cm^{-1}: 3317 (–OH), 1512 (phenyl). UV (MeOH) λ_{max}nm (logε): 281 (3.35), 227 (3.73). CD (MeOH) $\Delta\varepsilon$ (nm): +2.41 (279), +6.65 (225). ^{1}H-NMR (CD_3OD) δ: 2.22 (3H, s, N-CH_3), 3.10 (1H, td, J=4.2, 4.8 Hz, 2-H), 3.65 (1H, d, J=6.6 Hz, 5-H), 3.82 (1H, d, J=4.2, 11.7 Hz, 1-H), 3.85 (3H, s, O-CH_3), 3.86 (1H, dd, J=4.2, 11.7 Hz, 1-H), 3.96 (1H, dd, J=4.8, 6.6 Hz, 4-H), 4.01 (1H, t, J=4.8 Hz, 3-H), 6.81 (1H, dd, J=2.0, 8.3 Hz, 6′-H), 6.87 (1H, d, J=2.0 Hz, 2′-H), 6.90 (1H, d, J=8.3 Hz, 5′-H). ^{13}C-NMR (CD_3OD) δ: 35.1 (q, N-CH_3), 56.4 (t, C-1), 60.6 (q, O-CH_3), 71.1 (d, C-2), 75.4 (d, C-5), 79.6 (d, C-3), 85.1 (d, C-4), 112.5 (d, C-5′), 116.2 (d, C-2′), 121.3 (d, C-6′), 133.5 (s, C-1′), 147.7 (s, C-3′, 148.8 (s, C-4′). ESI-TOF-MS m/z: 270.1351 $[M+H]^+$ (Calcd for $C_{13}H_{20}NO_5$: 270.1341).

Codonopsinol B (**4**): Colorless amorphous gum: $[\alpha]_D$ −8° (c=0.45, MeOH). IR (ATR) ν_{max} cm^{-1}: 3313 (–OH), 1612, 1514 (phenyl). UV (MeOH) λ_{max}nm (logε): 276 (2.98), 226 (3.83). CD (MeOH) $\Delta\varepsilon$ (nm): +2.66 (277), +4.57 (220). ^{1}H-NMR (CD_3OD) δ: 2.16 (3H, s, N-CH_3), 3.06 (1H, td, J=4.0, 4.6 Hz, 2-H), 3.65 (1H, d, J=6.5 Hz, 5-H), 3.77 (3H, s, O-CH_3), 3.80 (1H, d, J=4.6, 11.5 Hz, 1-H), 3.84 (1H, dd, J=4.0, 11.5 Hz, 1-H), 3.92 (1H, dd, J=4.6, 6.5 Hz, 4-H), 4.00 (1H, t, J=4.6 Hz, 3-H), 6.89 (2H, d, J=8.5 Hz, 3′-H, 5′-H), 7.27 (2H, d, J=8.5 Hz, 2′-H, 6′-H). ^{13}C-NMR (CD_3OD) δ: 35.0 (q, N-CH_3), 55.7 (q, O-CH_3), 60.8 (t, C-1), 71.0 (d, C-2), 75.3 (d, C-5), 79.9 (d, C-3), 85.6 (d, C-4), 114.8 (d, C-3′, C-5′), 130.6 (d, C-2′, C-6′), 133.5 (s, C-1′), 160.7 (s, C-4′). ESI-TOF-MS m/z 254.1401 $[M+H]^+$ (Calcd for $C_{13}H_{20}NO_4$: 254.1392).

References

1) Wakana D., Kawahara N., Goda Y., *J. Nat. Med.*, **65**, 18–23 (2011).
2) Wang Z.-T., Xu G.-J., Hattori M., Namba T., *Shoyakugaku Zasshi*, **42**, 339–342 (1988).
3) Liu T., Liang W., Tu G., *Planta Med.*, **54**, 472–473 (1988).
4) Tsai T.-H., Lin L.-C., *Chem. Pharm. Bull.*, **56**, 1546–1550 (2008).
5) Ishida S., Okasaka M., Ramos F., Kashiwada Y., Takaishi Y., Kodzhimatov O. K., Ashurmetov O., *J. Nat. Med.*, **62**, 236–238 (2008).
6) Tsou E.-L., Chen S.-Y., Yang M.-H., Wang S.-C., Cheng T.-R., Cheng W.-C., *Bioorg. Med. Chem.*, **16**, 10198–10204 (2008).
7) Jagadeesh Y., Reddy J. S., Rao B. V., Swarnalatha J. L., *Tetrahedron*, **66**, 1202–1207 (2010).
8) Li C.-Y., Xu H.-X., Han Q.-B., Wu T.-S., *J. Chromatogr. A*, **1216**, 2124–2129 (2009).
9) Shibano M., Tsukamoto D., Masuda A., Tanaka Y., Kusano G., *Chem. Pharm. Bull.*, **49**, 1362–1365 (2001).
10) Shibano M., Kitagawa S., Nakamura S., Akazawa N., Kusano G., *Chem. Pharm. Bull.*, **45**, 700–705 (1997).
11) O'Hagan D., *Nat. Prod. Rep.*, **17**, 435–446 (2000).

Eight New Diterpenoids and Two New Nor-Diterpenoids from the Stems of *Croton cascarilloides*

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From the stems of *Croton cascarilloides*, eight new diterpenoids, named crotocascarins A–H (1–8), having a crotofolane skeleton were isolated along with two new nor-diterpenoids (9 and 10), named crotocascarins *α* and *β*, derived through rearrangement of the crotofolane skeleton. The structures of these compounds were elucidated by means of extensive one- and two-dimensional NMR spectroscopic analyses. The absolute structures of the diterpene moiety were determined by application of the circular dichroism (CD) rule for the *γ*-lactone ring. The relative structures of the two crotofolanes (1 and 2) and one rearranged compound (9) were confirmed by X-ray crystallographic analyses. Compounds 1, 2 and 9 possessed 2-methylbutyric acid in their molecules, the absolute configuration of which was found to be 2*S* by comparison of its HPLC behavior with that of an authentic sample. Therefore, the absolute structures of these crotocascarins (1, 2 and 9) were unambiguously determined. The absolute structures of crotofolanes are reported for the first time in this paper.

Key words *Croton cascarilloides*; Euphorbiaceae; crotocascarin; crotofolane; diterpenoid

Crotofolane-type diterpenoids have fused 5-, 6- and 7-membered rings, and are expected to be biosynthesized from cembranes *via* lathyrane through cross annular cyclization.[1] In 1975, this type of diterpenoid was first found in Jamaican *Croton* species, *C. corylifolius* LAMARCK as crotofolin A as shown in Fig. 1.[1,2] Only two other species, Kenyan *C. dichogamus* PAX[3] and Congolese *C. haumanianus* J. LÉONARD,[4] were also found to contain rare crotofolanes. Genus *Croton* (Euphorbiaceae) comprises about 600 species and is distributed in tropical areas of both hemispheres. Croton oil obtained from seeds of *C. tiglium* LINNÉ has a strong purgative effect and the occurrence of cocarcinogenic agents; fatty acid esters of diterpene phorbol in this plant has attracted our interest to investigate this genus.[5] *C. cascarilloides* RÄUSCHEL is an evergreen shrubby tree that grows on elevated coral reefs of the Okinawa Islands, Taiwan, southern China, the Malay Peninsula and Malaysia. Leaves are oblong-lanceolate to oblong-oval, and their undersurface is covered by shiny white ramenta.[6] Our phytochemical investigation of the stems (14.5 kg) of *C. cascarilloides* collected in the Okinawa Islands led to the isolation of eight new crotofolane-type diterpenoids, given trivial names crotocascarins A–H (**1**–**8**), and two new nor-diterpenoids with a new skeleton, given trivial names crotocascarins *α* and *β* (**9**, **10**). The absolute configuration of crotofolanes was first determined in this study.

Results and Discussion

Using several types of chromatography, diterpenoids (**1**–**8**) and nor-diterpenoids (**9**, **10**) were isolated from a CH_2Cl_2-soluble fraction prepared from the MeOH extract of the stems of *C. cascarilloides* (Fig. 2).

Crotocascarin A (**1**), $[\alpha]_D^{26}$ +16.4, was isolated as colorless rods and its elemental composition was determined to be $C_{25}H_{32}O_7$ by high-resolution (HR)-electrospray ionization (ESI)-mass spectrometry. The IR spectrum showed absorption bands for ester carbonyl and lactone carbonyl groups (1763, 1739 cm^{-1}), and double bonds (1650 cm^{-1}). In the ^{1}H-NMR spectrum, signals for two singlet methyls, two doublet methyls and one triplet methyl together with ones for two olefinic protons (δ_H 5.06, 5.09) and two oxygenated methine protons (δ_H 3.17, 5.48) were observed (Table 1). The ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) spectra (Fig. 3) together with one-dimensional ones indicated the presence of a 2-methylbutanoic acid moiety, the remaining 20 ^{13}C-NMR signals comprising those of three methyls, three methylenes, five methines, one tetra- and one disubstituted double bonds, three oxygenated tertiary carbons, one carbonyl carbon and one hemiketal carbon (δ_C 107.5) (Table 2). The ten degrees of unsaturation, based on the results of HR-ESI-MS, other than two carbonyl groups and two double bonds, required six ring systems in the skeleton. Precise inspection of two-dimensional NMR spectra led to the conclusion that compound **1** was a diterpenoid with

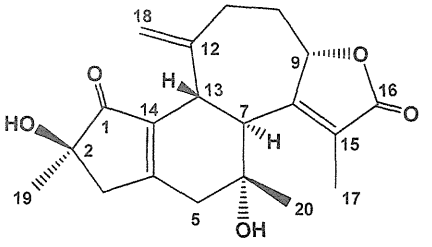

Fig. 1. Structure of Crotofolin A, Isolated from *C. corylifolius*

The authors declare no conflict of interest.

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Table 1. ^{1}H-NMR Spectroscopic Data for Crotocascarins A–H (**1**–**8**), and α and β (**9**, **10**) (400 MHz, $CDCl_3$)

H	1	2	3	4	5	6	7	8	9	10
1	5.48 d 5	5.38 d 5	5.34 d 5	5.49 d 5	5.43 d 5	5.48 d 5	5.39 d 5	5.35 d 4	5.78 d 5	5.82 d 5
2	2.18 m	2.18 dqdd 8, 7, 7, 5	2.18 m	2.21 m	2.16 m	2.20 m	2.18 m	2.18 m	2.22 m	2.22 m
3	2.47 dd 14, 7	2.49 dd 14, 8	2.50 dd 13, 7	2.48 dd 14, 7	2.48 dd 14, 7	2.47 dd 14, 7	2.50 dd 14, 7	2.50 dd 14, 7	1.56 dd 14, 10	1.52 dd 14, 10
	1.65 dd 14, 10	1.70 dd 14, 10	1.72 dd 13, 10	1.64 dd 14, 10	1.69 dd 14, 10	1.65 dd 14, 10	1.70 dd 14, 10	1.71 dd 14, 10	2.34 dd 14, 7	2.36 dd 14, 7
4	—	—	—	—	—	—	—	—	—	—
5	3.17 s	3.19 s	3.20 s	3.15 s	3.18 s	3.16 s	3.19 s	3.20 s	4.39 s	4.36 s
6	—	—	—	—	—	—	—	—	—	—
7	2.99 dd 13, 1	3.06 br d 12	3.00 dd 12, 1	3.10 d 13	2.95 dd 12, 1	2.99 d 13	3.06 d 13	2.99 dd 12, 1	2.92 d 13	3.01 d 13
8	—	—	—	—	—	—	—	—	—	—
9	—	5.14 ddd 13, 4, 2		4.85 dddd 11, 4, 2, 2	—	—	5.14 brd 11	—	—	—
10	2.46 m	2.73 ddd 13, 4, 4	2.76 dd 14, 3	2.51 m	3.04 dd 16, 2	2.44 m	2.73 ddd 14, 4, 4	2.75 dd 15, 3	2.07 dd 15, 10	2.17 m
	1.57 ddd 13, 13, 5	1.27 ddd 13, 13, 4	1.70 dd 14, 3	1.17 m	1.45 ddd 16, 5, 2	1.58 ddd 13, 13, 5	1.30 brd 14	1.71 ddd 15, 3, 2	2.46 dd 15, 7	2.36 m
11	2.48 m	4.53 ddd 4, 4, 2	4.61 dd 3, 3	2.52 m	4.43 ddd 5, 2, 2	2.44 m	4.52 dd 3, 3	4.60 ddd 3, 3, 2	4.17 brdd 10, 7	2.09 m
	2.14 ddd 13, 13, 5			2.44 m		2.11 ddd 13, 13, 5				2.17 m
12	—	—	—	—	—	—	—	—	—	—
13	3.05 d 13	3.15 d 13	3.45 d 12	2.56 d 13	3.52 d 12	3.04 d 13	3.15 d 13	3.45 d 12	3.11 brd 13	2.99 brd 13
14	—	—	—	—	—	—	—	—	—	—
15	—	—	—	—	—	—	—	—	—	—
16	—	—	—	—	—	—	—	—		
17	1.90 3H d 1	1.90 3H brs	1.89 3H brs	1.91 3H brs	1.88 3H d 1	1.91 3H d 1	1.90 3H d 1	1.89 3H d 1	2.39 3H, s	2.39 3H s
18	5.09 s	5.20 s	5.28 s	5.09 s	5.23 s	5.09 s	5.21 s	5.28 s	5.23 brs	4.92 s
	5.06 brs	5.17 s	5.23 brs	5.07 brs	5.17 brs	5.06 brs	5.16 brs	5.23 brs	5.39 s	5.20 brs
19	0.92 3H d 7	0.98 3H d 7	1.00 3H d 7	0.92 3H d 7	0.96 d 7	0.91 3H d 7	0.97 3H d 7	0.99 3H d 7	0.92 3H, d 7	0.90 3H d 7
20	1.17 3H s	1.06 3H s	1.17 3H s	1.07 3H s	1.14 3H s	1.17 3H s	1.06 3H s	1.17 3H s	1.27 3H, s	1.29 3H s
1′	—	—	—					—	—	—
2′	2.43 m	2.49 qdd 7, 7, 7	2.49 qdd 7, 7, 7	2.49 m	2.47 qdd 7, 7, 7	2.62 septet 7	2.65 septet 7	2.66 septet 7	2.38 m	2.36 m
3′	1.72 dqd 14, 7, 7	1.74 dqd 14, 7, 7	1.72 dqd 14, 7, 7	1.73 dqd 14, 7, 7	1.75 dqd 14, 7, 7	1.21 3H s	1.21 3H s	1.20 3H s	1.43 dqd 14, 7, 7	1.42 dqd 14, 7, 7
	1.48 dqd 14, 7, 7	1.50 dqd 14, 7, 7	1.49 dqd 14, 7, 7	1.49 dqd 14, 7, 7	1.50 dqd 14, 7, 7	1.21 3H s	1.20 3H s	1.19 3H s	1.68 dqd 14, 7, 7	1.64 dqd 14, 7, 7
4′	0.94 3H t 7	0.93 3H t 7	1.19 3H t 7	0.94 3H t 7	0.93 3H t 7				0.90 3H t 7	0.89 3H t 7
5′	1.19 3H d 7	1.17 3H d 7	1.17 3H d 7	1.18 3H d 7	1.19 3H 6				1.12 3H d 7	1.10 3H d 7
$-OCH_3$					3.57 3H s					
9-OH						3.49 brs				
11-OH		2.44 dd 2, 2			2.42 dd 2, 2			3.05 dd 2, 2		2.26 d 6

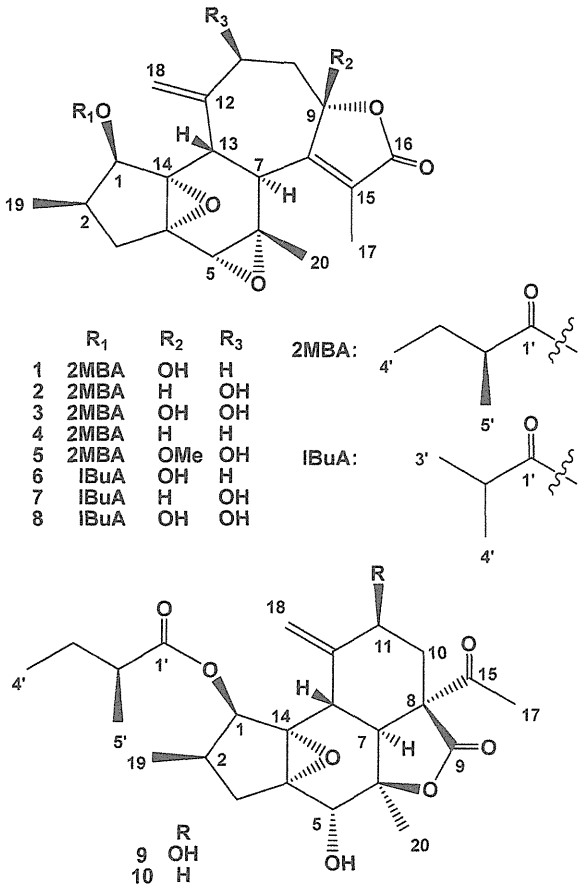

Fig. 2. Structures of New Compounds Isolated

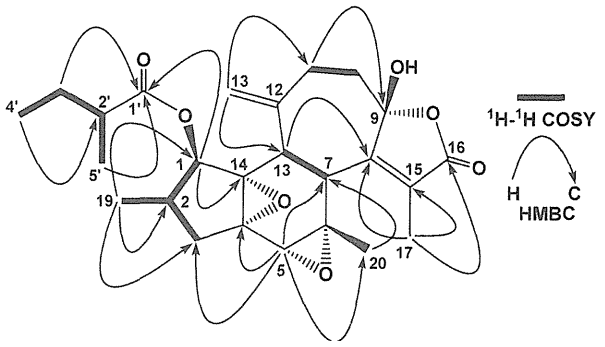

Fig. 3. ^{1}H–^{1}H COSY and Selected HMBC Correlation of **1**

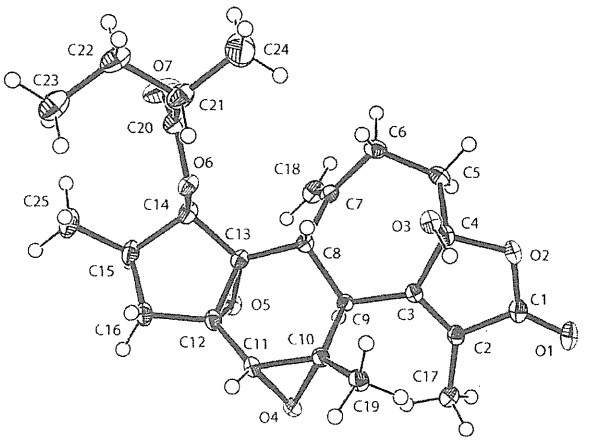

Fig. 4. ORTEP Drawing of **1**

The crystal structure has crystallographic numbering.

an unusual carbon skeleton (Fig. 2). To confirm this, X-ray crystallographic analysis of **1** was performed and the relative stereostructure of **1** was established to be that of a derivative of crotofolane-type diterpenoid (Fig. 4). The positive Cotton effect observable in the circular dichroism (CD) spectrum [$\Delta\varepsilon$ +3.41 (251)] empirically indicated the absolute configuration at the 9-position of the α,β-unsaturated γ-lactone ring was *S*,[7,8] and chirality analysis of the 2-methylbutanoic acid moiety by HPLC with an optical rotation detector established the absolute configuration of **1** to be shown in Fig. 2. This is the first report of the absolute structure of a crotofolane diterpene being disclosed and the absolute configuration of the α,β-unsaturated γ-lactone portion, presumed based on the empirical rule for the CD spectra, was proved to be correct.

Crotocascarin B (**2**), $[\alpha]_D^{26}$ +81.8, was isolated as colorless plates and its elemental composition was determined to be $C_{25}H_{32}O_7$ by HR-ESI-MS. NMR spectroscopic data indicated that **2** was a similar compound to **1**, except for the disappearance of the hemiketal signal, instead of which, two oxymethine signals (δ_H 5.14 on δ_C 78.4 and 4.53 on 72.7) were observed. Since these oxymethine protons were correlated in the ^{1}H–^{1}H COSY spectrum through methylene protons on C-10, the planar structure of **2** was established to be as shown in Fig. 2. To confirm this, X-ray crystallographic analysis of **2** was performed and the relative stereostructure of **2** was established to be that of a derivative of crotofolane-type diterpenoid[9] (Fig. 5). The positive Cotton effect observable in the CD spectrum [$\Delta\varepsilon$ +1.36 (249)] indicated the absolute configuration at the 9-position of the α,β-unsaturated γ-lactone ring was also *S*, and chirality analysis of the 2-methylbutanoic acid moiety by HPLC with an optical rotation detector established the absolute structure of **2** to be as shown in Fig. 2.

Crotocascarin C (**3**), $[\alpha]_D^{24}$ +88.9, and crotocascarin D (**4**), $[\alpha]_D^{24}$ +2.6, were also similar compounds to the aforementioned ones with the respective elemental compositions of $C_{25}H_{32}O_8$ and $C_{25}H_{32}O_6$. In the NMR spectra of **3** and **4**, a hemiketal carbon signal (C-9: δ_C 106.4) and an oxygenated carbon signal (C-11: δ_C 73.7) with δ_H 4.61 were observed in the former, and an oxygenated carbon signal (C-9: δ_C 82.2) and a methylene signal (C-11: δ_C 36.2) in the latter. A similar coupling pattern fot H-11 in **3** to that of **2** placed the hydroxy group at the 11-position in a β-orientation. Since the ^{1}H–^{1}H COSY spectrum of the former exhibited the proton chain from H_2-10 to H-11, along with the observation of a hemiketal carbon in the ^{13}C-NMR spectrum, and that of the latter one from H-9 to H_2-11 through H_2-10, their structures were established to be as shown in Fig. 2. A similar coupling pattern for H-11 in **3** to that of **2** placed the hydroxy group at the 11-position in a β-orientation and positive Cotton effects at 252 nm ($\Delta\varepsilon$ +4.47, +1.17, respectively) substantiated that the absolute configurations of the diterpene moieties were the same as those of aforementioned compounds. The absolute configuration of 2-methylbutanoic acid in **3** and **4** must also be the same as that in **1** and **2**, judging from the ^{13}C-NMR chemical shifts of