ORGANIC LETTERS

2013
Vol. 15, No. 15
3888–3890

First Synthesis of a Stable Isotope of Ochratoxin A Metabolite for a Reliable Detoxification Monitoring

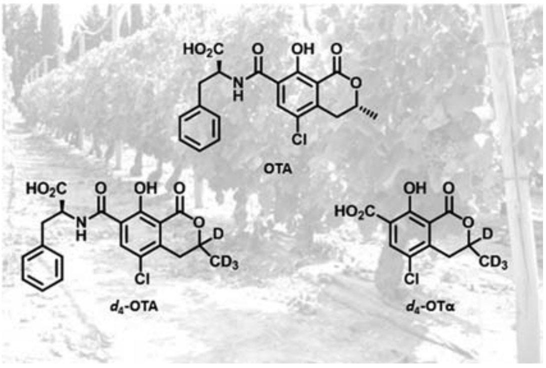
Anaïs Bouisseau,[†,‡] Aurélie Roland,[‡] Florence Reillon,[‡] Rémi Schneider,[‡,§] and Florine Cavelier*[,†]

IBMM, UMR-CNRS-5247, Universités Montpellier I and II, Place Eugène Bataillon,
34095 Montpellier, Cedex 5, France, Nyseos, Bâtiment 28, 2 place Pierre Viala,
34060 Montpellier Cedex 1, France, IFV UMT Qualinnov, 2 place Pierre Viala,
34060 Montpellier Cedex 1, France

florine@univ-montp2.fr

Received June 10, 2013

ABSTRACT



Due to its toxicity and presence in numerous food products, Ochratoxin A (OTA) has drawn attention for decades. This article summarizes the first synthesis of a labeled analogue of Ochratoxin α (OTα), one of the main products generated by the metabolization of OTA by microorganisms. This synthesis also led to a new labeled analogue of OTA with the deuteration located on the dihydroisocoumarin moiety allowing thus both the accurate quantification of OTA and OTα and the establishing of a reliable detoxification rate.

The mycotoxin Ochratoxin A (OTA) (**1**, Figure 1) was first isolated by van der Merwe et al. in 1965 from a South African culture of *Aspergillus ochraceus* grown on a sterile maize meal.[1] In 1992, Marquardt and Frohlich assessed the general toxicity of this toxin.[2] Since, OTA has drawn the attention of the International Agency for Research on Cancer and belongs to the group 2B.[3]

OTA has been consequently classified as a possible human carcinogenic substance and presents also many toxic effects on animals such as carcinogenicity in mice and rats kidneys and livers, hepatotoxicity, teratogenicity, immunosuppressivity, and nephrotoxicity.[4−7]

This mycotoxin can contaminate different food products such as coffee, wheat, grapes, and wine.[8,9] The presence of OTA in wine is mainly due to the fungal contamination of grapes by *Aspergillus carbonarius*, and the occurrence depends on its geographic origin and on the type of wine.[10,11]

[†] IBMM.
[‡] Nyseos.
[§] IFV UMT Qualinnov.

(1) van der Merwe, K. J.; Steyn, P. S.; Fourie, L.; Scott, D. B.; Theron, J. J. *Nature* **1965**, *205*, 1112–1113.

(2) Marquardt, R. R.; Frohlich, A. A. *J. Anim. Sci.* **1992**, *70*, 3968–3988.

(3) IARC, *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* **1993**, *56*, 489–521.

(4) Krogh, P. *Food Chem. Toxicol.* **1992**, *30*, 213–224.

(5) Bendele, A. M.; Carlton, W. W.; Krogh, P.; Lillehoj, E. B. *J. Natl. Cancer Inst.* **1985**, *75*, 733–742.

(6) Chopra, M.; Link, P.; Michels, C.; Schrenk, D. *Cell. Biol. Toxicol.* **2010**, *26*, 239–254.

(7) Haubeck, H. D.; Lorkowski, G.; Kolsch, E.; Roschenthaler, R. *Appl. Environ. Microbiol.* **1981**, *41*, 1040–1042.

(8) Pittet, A. *Rev. Med. Vet.* **1998**, *149*, 479–492.

(9) Jørgensen, K. *Food Addit. Contam.* **2005**, *22*, 26–30.

(10) Fabiani, A.; Corzani, C.; Arfelli, G. *Talanta* **2010**, *83*, 281–285.

10.1021/ol401630t

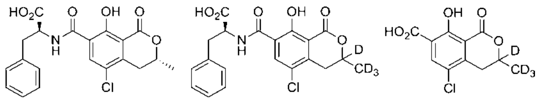
The synthesis of the four diastereoisomers of OTA has been described in order to assess the cytotoxicity of each chiral compound separately.[12] Another publication describes the synthesis of natural and d_5-OTA with the deuteration on the phenylalanine moiety.[13]

As contamination of crops by *Aspergillus sp.* is difficult to avoid, due to the lack of infestation predictive models, the food industry is looking for curative tools.

In wine, different ways of detoxification have been described, either by physical, chemical, or microbiological means.[14] The problem remains to determine the structure and the potential toxicity of the degradation products. For thermal processes, such as in coffee, OTA was reported to be transformed into 14-(*R*)-OTA by isomerization and 14-decarboxy-OTA by decarboxylation, both less toxic.[15] For microbiological processes, not only some industrial yeast strains but also lactic acid bacteria or filamentus fungi are able to degrade OTA into ochratoxin α (OTα),[14,16,17] which is less toxic.[18,19] But it cannot be excluded that other potentially toxic degradation products could be produced according to a microbiological process. Abrunhosa et al. described the two main metabolization pathways involved for OTA when using microorganisms. The first is the transformation of OTA into OTα which represents a detoxification process because OTα is much less toxic.[20] The second main product is the compound obtained after hydrolysis of the OTA lactone ring, but this resulting opened-lactone molecule showed a high *in vivo* toxicity.[18]

Thus, labeled OTα together with OTA could be of interest to establish a precise quantification of the hydrolysis rate of the amide bond between phenylalanine and OTα. By extension, this quantification of both OTA and OTα after a microbiological process could represent a reliable method for the determination of a real detoxification rate since it is not only the disappearance of OTA which will be studied but also the formation of the non-toxic metabolization product, OTα.

In order to perform these quantifications accurately, Stable Isotope Dilution Assay appears as a good alternative, and thus, syntheses of deuterated OTA and OTα are required for their use as internal standards.



1 2 3

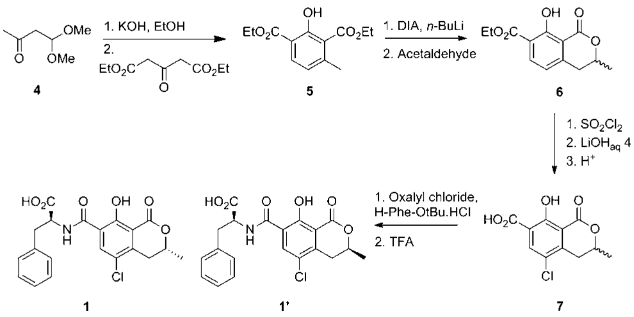
Figure 1. Natural OTA (**1**), d_4-OTA (**2**), and d_4-OTα (**3**).

Our study presents the synthesis of natural OTA (**1**, Figure 1) and of a new isotope d_4-OTA (**2**, Figure 1), with the deuteration located on the dihydroisocoumarin moiety. We also describe the first synthesis of d_4-OTα (**3**, Figure 1) which could be used for degradation studies as mentioned above.

The synthesis of OTA was previously described using Covarrubias–Zúñiga's method,[21] which involves a reaction between the sodium salt of dimethyl 3-oxopentanedioate and but-2-ynal. We chose to synthesize the diethyl ester **5** instead of the dimethyl ester via Antus et al.'s method.[22] Thus, the potassium salt was prepared by alkali-catalyzed cleavage of 4,4-dimethoxybutan-2-one **4** which was then reacted with diethyl 3-oxopentanedioate to afford **5**. Then, following Cramer et al.'s synthesis and as shown in Scheme 1, the condensation step with acetaldehyde led to the intermediate **6** as an enantiomeric mixture in high yields.[12] Racemic OTα **7** was prepared by chlorination and saponification of **6**.

To achieve the synthesis of OTA **1** from OTα **7**, the next step was the introduction of a phenylalanine moiety (Scheme 1). Among all the conditions tested, including BOP and HATU as coupling agents, the use of oxalyl chloride appeared to be the most efficient for coupling OTα **7** with L-phenylalanine *tert*-butyl ester.[23,24] The *tert*-butyl group was then removed using TFA.[25] This deprotection step led to a mixture of natural OTA **1** and its diastereoisomer **1′**.

Scheme 1. Synthesis of Natural OTA **1** and Its Diastereoisomer **1′**



---

(11) Otteneder, H.; Majerus, P. *Food Addit. Contam.* **2000**, *17*, 793–798.

(12) Cramer, B.; Harrer, H.; Nakamura, K.; Uemura, D.; Humpf, H.-U. *Biorg. Med. Chem.* **2010**, *18*, 343–347.

(13) Gabriele, B.; Attya, M.; Fazio, A.; Di Donna, L.; Plastina, P.; Sindona, G. *Synthesis* **2009**, *11*, 1815–1820.

(14) Quintela, S.; Villaran, M. C.; de Armentia, I. L.; Elejalde, E. *Food Control* **2013**, *30*, 439–445.

(15) Cramer, B.; Konigs, M.; Humpf, H. U. *J. Agric. Food. Chem.* **2008**, *56*, 5673–5681.

(16) Cecchini, F.; Morassut, M.; Moruno, E. G.; Di Stefano, R. *Food Microbiol.* **2006**, *23*, 411–417.

(17) Peteri, Z.; Teren, J.; Vagvolgyi, C.; Varga, J. *Food Microbiol.* **2007**, *24*, 205–210.

(18) Xiao, H.; Madhyastha, S.; Marquardt, R. R.; Li, S. Z.; Vodela, J. K.; Frohlich, A. A.; Kemppainen, B. W. *Toxicol. Appl. Pharmacol.* **1996**, *137*, 182–192.

(19) Stander, M. A.; Steyn, P. S.; van der Westhuizen, F. H.; Payne, B. E. *Chem. Res. Toxicol.* **2001**, *14*, 302–304.

(20) Abrunhosa, L.; Paterson, R. R. M.; Venancio, A. *Toxins* **2010**, *2*, 1078–1099.

(21) Covarrubias-Zúñiga, A.; Ríos-Barrios, E. *J. Org. Chem.* **1997**, *62*, 5688–5689.

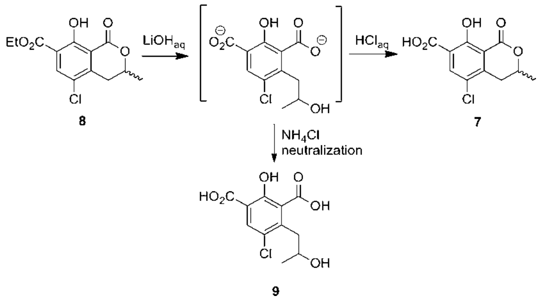
(22) Antus, S.; Boross, F.; Nógrádi, M. *Liebigs Ann.* **1978**, *1978*, 107–117.

(23) Adams, R.; Ulich, L. H. *J. Am. Chem. Soc.* **1920**, *42*, 599–611.

(24) Montalbetti, C. A. G. N.; Falque, V. *Tetrahedron* **2005**, *61*, 10827–10852.

(25) Bryan, D. B.; Hall, R. F.; Holden, K. G.; Huffman, W. F.; Gleason, J. G. *J. Am. Chem. Soc.* **1977**, *99*, 2353–2355.

Scheme 2. Saponification Step



As shown in Scheme 2, it should be noted that HPLC and LC/MS monitorings showed that the saponification step was actually a disaponification, and the lactone **7** was reformed under acidic conditions. Indeed, while the chlorinated intermediate **8** presents a retention time of 2.01 min, we obtained, after lithium hydroxide addition and ammonium chloride neutralization, the opened lactone form **9** of OTα with a retention time of 1.19 min ($[\mathrm{MH}^+] = 275.1$), which led to compound **7** ($[\mathrm{MH}^+] = 257.2$) with a retention time of 1.48 min under acidic conditions. Compound **9** unfortunately could not be purified by silica column because of its high polarity or by preparative HPLC because of its instability under acidic conditions.

Using the appropriate labeled reagent, the same procedure was used to prepare d_4-OTα **3** and d_4-OTA **2** as a mixture of deuterated enantiomers and diastereoisomers respectively. Those three compounds were fully characterized after separation of d_4-OTA diastereoisomers.

A separation of the nondeuterated diastereoisomers on preparative HPLC allowed us to isolate (−)-OTA **1** (natural form).

Since (+)-OTA does not occur in food products, the mixture of deuterated diastereoisomers can be directly used as an internal standard for OTA quantification in wine samples upon the condition that the same diastereomeric mixture be used for calibration. The analysis study of wine samples was based on LC/MS. Using a mixture of deuterated diastereoisomers, we observed an overlapping of (−)-OTA and one of the diastereoisomers of d_4-OTA by liquid chromatography. But since the quantification was determined by mass spectrometry, the detection was based on the 4 Da shift in the molecular mass corresponding to the introduction of four deuteriums.

In conclusion, we designed a synthetic pathway that led to both d_4-OTα and d_4-OTA. We achieved the synthesis of a new labeled analogue of natural OTA bearing the deuteration on the dihydroisocoumarin moiety of the molecule, opposite to phenylalanine. In addition, our strategy allowed us to produce for the first time a labeled analogue of OTα, which can be used for establishing a reliable detoxification rate.

Acknowledgment. Raymond Baumes (INRA) is kindly thanked for helpful discussions at the beginning of the study. France AgriMer and Région Languedoc Roussillon are thanked for their financial support.

Supporting Information Available. Experimental procedures for the preparation of natural and deutarated OTA and characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.