Synthesis of Isotopically Labeled *Fusarium* Mycotoxin $^{13}C_2$-Moniliformin [1-Hydroxycyclobut-1-ene-3,4-dione]

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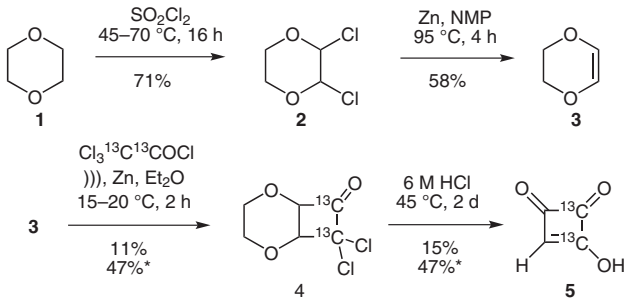
Abstract: The total synthesis of isotopically labeled [$^{13}C_2$]-1-hydroxycyclobut-1-ene-3,4-dione (moniliformin) a fungal toxic secondary metabolite to be used as internal standard for mycotoxin analysis is described. The synthesis proceeds in four steps starting from 1,4-dioxane, which was converted to 2,3-dihydro-1,4-dioxine followed by a [2+2]-cycloaddition with trichloroacetyl chloride-1,2-$^{13}C_2$ as ^{13}C source. The $^{13}C_2$-labeled cyclobutanone precursor was transformed to [$^{13}C_2$]-moniliformin by acid-catalyzed hydrolysis. The successful incorporation of two ^{13}C atoms was proven by detailed NMR and mass spectrometric studies of labeled moniliformin and its precursor.

Key words: *Fusarium*, moniliformin, [2+2] cycloaddition, stable isotope dilution analysis, HPLC–MS/MS

Moniliformin (MON) is a toxic secondary fungal metabolite in cereals and maize worldwide,[1] which is produced by a number of *Fusarium* species, including the common corn pathogen *F. avenaceum*, *F. proliferatum*, *F. subglutinans*, *F. tricinctum*, and *F. verticillioides*.[2] Naturally it occurs as sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione and was first isolated and characterized by Cole et al. in 1973.[3,4] Toxicity studies with different animal species indicate that MON is a potent cardiotoxin.[5,6] Thiel et al. suggested that the molecular mechanism for the toxic effect of MON involves selective inhibition of mitochondrial pyruvate and α-ketoglutarate oxidations.[7]

Due to the potential health risk for humans and animals posed by MON, the establishment of fast and reliable methods for the detection of this mycotoxin in different food and feed as well as physiological samples is of great importance. For this purpose the use of high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (HPLC–MS/MS) is the method of choice. Advantages of HPLC–MS/MS analysis are the high selectivity and sensitivity. Thus this technique has a wide application in food and feed analysis. However, the use of HPLC–MS/MS is mostly limited by the influence of coeluting matrix compounds on the ionisation of the analyte.[8] To overcome this limitation the stable isotope dilution analysis by using isotopically labeled standards is often applied.[9] However, until now no isotopically labeled MON is available and no strategy for the introduction of stable isotopes has been proposed. Therefore our aim was to synthesize isotopically labeled MON to be used as internal standard in stable isotope dilution analysis. So far several synthetic routes to MON have been reported and all rely on the generation of cyclobutane derivatives as precursors.[10] The most recent synthesis was reported by Fétizon et al. in 1990 based on a [2+2] cycloaddition of 2,3-dihydro-1,4-dioxine and dichloroketene via formation of a four-membered ring precursor in 34% yield.[11] By hydrolysis of the cyclobutanone precursor Fétizon and coworkers were able to obtain MON in 82% yield. Based on this straightforward approach we developed a modified strategy for the first preparation of isotopically labeled MON by using trichloroacetyl chloride-1,2-$^{13}C_2$ as ^{13}C source for isotope introduction.



Scheme 1 Synthetic route for the preparation of $^{13}C_2$-moniliformin (* yields for unlabeled compounds)

Since MON is known to be among the strongest naturally occurring organic acid ($pK_a = 0.88$), the two hydrogen atoms in the molecule would undergo rapid hydrogen–deuterium atom exchange in methanol or water, such that deuterium-labeled MON would not be useful as internal standard.[12,13] To overcome this challenge we decided to synthesize instead a ^{13}C-labeled standard.

The synthesis started with the preparation of 2,3-dihydro-1,4-dioxine (**3**) for which several procedures are published.[14,15] We synthesized **3** in two steps starting from 1,4-dioxane **1** (Scheme 1) following a modified procedure by Shinohara et al.[16] Firstly 2,3-dichloro-1,4-dioxane (**2**) was generated by chlorination of **1** with sulfuryl chloride

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(71%). In a reductive dehalogenation using activated Zn **2** was reacted in different polar solvents to give **3**. After isolation by distillation, **3** could be obtained in 58% yield when NMP was employed as solvent. Using DMF or HMPA the yield was 18% and 10%, and in the case of DMSO no reaction occurred.[17]

The key step in the reaction pathway is the [2+2] cycloaddition between **3** and dichloroketene which is generated in situ by a reductive dehalogenation of trichloroacetyl chloride with activated zinc. At this stage the isotope labeling can be introduced by application of trichloroacetyl chloride-1,2-$^{13}C_2$. The optimization of the [2+2] cycloaddition reaction using unlabeled trichloroacetyl chloride is shown in Table 1. Fétizon et al. yielded 34% of 7,7-dichloro-2,5-dioxabicyclo[4.2.0]octan-8-one (**4**), and they suggested that the moderate yield is due to the low reactivity of **3**.[11] However, several attempts to reproduce the described results failed, and we were only able to observe traces of **4** (determined by ^{1}H NMR). By addition of molecular sieves to the reaction mixture, the yield of **4** could be raised to 19%. Nevertheless, the obtained yields were not sufficient regarding the application of expensive trichloroacetyl chloride-1,2-$^{13}C_2$. The use of Zn–Cu did not afford any increase of the yield. Shortening the reaction time to two hours using activated zinc leads to an improved yield of 47%. With this optimized reaction conditions we carried out the [2+2] cycloaddition with trichloroacetyl chloride-1,2-$^{13}C_2$ and obtained the desired $^{13}C_2$-cycloproduct **4** in a yield of 11%.

The comparison of the ^{1}H NMR and ^{13}C NMR spectra of labeled and unlabeled **4** proved the successful incorporation of the two ^{13}C atoms. Figure 1 displays the ^{1}H NMR and ^{13}C NMR spectra of labeled (A, C) and unlabeled (B, D) **4**. The two analogues vary in their ^{1}H NMR (see Figure 1, A and B) spectra regarding the signals at δ = 4.40 ppm (H-4) and δ = 5.36 ppm (H-5). Unlabeled **4** shows two doublets for these signals whereas labeled **4** exhibits splitting for both signals due to the coupling with ^{13}C-labeled C-8. HMBC experiments of unlabeled and labeled **4** surprisingly revealed the absence of a ^3J coupling between H-5 and C-7 and ^2J coupling between H-4 and C-7. The ^{13}C NMR spectrum of unlabeled **4** (see Figure 1, D) shows six signals whereas in the spectrum of the labeled analogue (see Figure 1, C) only two significant signals of the two incorporated ^{13}C atoms at δ = 192.77 ppm (C-8) and δ = 81.47 ppm (C-7) appear showing a coupling of 30.9 Hz. Also the comparison of the exact masses and mass spectrometric fragmentation patterns of labeled (see Figure 2, A) and unlabeled (see Figure 2, B) **4** proved the successful incorporation of two ^{13}C atoms. The pseudomolecular mass $[M + H]^+$ = 198.9834 is two mass units higher for labeled **4**.

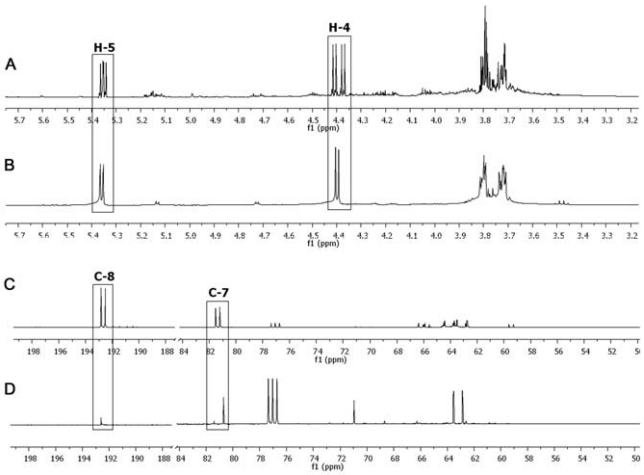

Figure 1 400 MHz ^{1}H NMR spectrum of labeled (A) and unlabeled (B) 7,7-dichloro-2,5-dioxabicyclo[4.2.0]octan-8-one (**4**); 100 MHz ^{13}C NMR spectrum of labeled (C) and unlabeled (D) 7,7-dichloro-2,5-dioxabicyclo[4.2.0]octan-8-one (**4**)

Hydrolysis of **4** using 6 M HCl following the reported conditions afforded MON in less than 10% yield. After optimization, we were able to obtain **5** in 47% yield by increasing the reaction time up to two days and lowering the temperature to 45 °C.

The purification of MON (**5**) proved to be challenging due to the high polarity and the ionic nature of this compound.[18] Prefractioning on a C_{18}-cartridge (removal of HCl) and purification by preparative HPLC using a semi-preparative Gemini-C6-Phenyl Column afforded **5** in the desired purity. Hydrolysis of $^{13}C_2$-labeled **4** provided 7 mg (15%) $^{13}C_2$-MON with a purity of >95% (^{1}H NMR) and an isotope purity of 99% (HPLC–MS). The incorporation of two ^{13}C atoms was proved by the exact mass of the

Table 1 Optimization of the [2+2] Cycloaddition between 2,3-Dihydro-1,4-dioxine and Unlabeled/Labeled Trichloroacetyl Chloride

Entry	Catalyst (1.2 equiv)	Addition of 4 Å MS	Time of Cl_3CCOCl addition (h)	Sonication after addition (h)	Yield of **4** (%)[a]
1	Zn	–	4	0.15	trace (34)[b]
2	Zn	+	4	0.15	19
3	Zn	+	0.3	2	47 (11)[c]
4	Zn–Cu	+	0.3	2	23
5	Zn–Cu	+	4	0.15	18

[a] Isolated yields. Corresponding to trichloroacetyl chloride.
[b] Reported yield (Fétizon et al.).
[c] Yield for [^{13}C-7, ^{13}C-8]-7,7-dichloro-2,5-dioxabicyclo[4.2.0]octan-8-one.

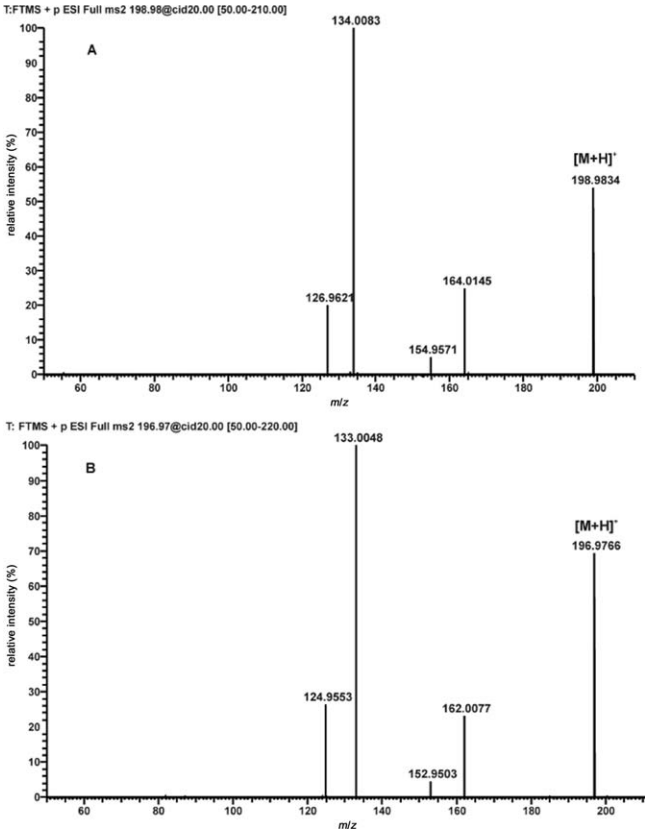

Figure 2 ESI-HRMS/MS product ion spectra obtained by CID (20V) of the molecular ion *m/z* = 198.9834 (labeled **4**, A) and *m/z* = 196.9766 (unlabeled **4**, B)

pseudomolecular ion $[M - H]^-$ which is two mass units higher than that of unlabeled MON.

This is the first report of the synthesis of $^{13}C_2$-MON allowing access to an internal standard for stable isotope dilution analysis of MON in complex food and feed matrices using HPLC–MS/MS techniques.

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

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(17) Compound **3** never distilled over separately neither at atmospheric nor at reduced pressure. Distillation was only possible as azeotropic mixture after the addition of H_2O.
(18) Purification by continuous liquid–liquid extraction with Et_2O did not give moniliformin in sufficiently pure grade (NMR).