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Observations on Fragmentation Pathway of Farinomalein and its Isomers by Structural Investigation Using LC-MS/MS

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Abstract : Farinomalein is a maleimide-bearing compound well known for its anti-fungal activity. In the present study, synthesis of farinomalein is achieved via Stobbe condensation followed by Haval-Argade contrathermodynamic rearrangement. Kinetically driven Stobbe condensation followed by condensation with beta-alanine reveals formation of two isomers of farinomalein. This article describes application of LC-MS/MS in structure elucidation of farinomalein **1** and its isomers **2** and **3** encountered in its synthesis. The proposed distinct fragmentation pathway is supported by rational organic reaction mechanism. These fragmentation pathways are significant for analytical method development of farinomalein in near future. The structures of farinomalein **1** and its isomers **2** and **3** have been assigned undisputedly.

Keywords : Maleimide, Stobbe condensation, Haval-Argade contrathermodynamic rearrangement, Isofarinomalein

Introduction

N-substituted maleimides are found in nature and prehistorically known for their spectrum of bio-activities. Farinomalein **1** is a maleimide containing compound, recently isolated from entomopathogenic fungus *P. farinosus* HF599 by Nihira *et al*. in the year 2009.[1] Farinomalein **1** has appreciable anti-fungal activity with Minimum Inhibition Concentration value (5 μg/disk) in comparison to positive control, amphotericin B having MIC value (10 μg/disk) against the plant pathogen *Phytophthora sojae*. The structure of the farinomalein **1** was supported by ESI-TOF-MS, ^{1}H-NMR, ^{13}C-NMR and related experiments. The first synthesis of farinomalein is achieved via γ-hydroxybutenolide.[2] The second enhanced synthesis of farinomalein is reported from ethyl 3-methyl-2-oxobutyrate[3] as a starting compound, Lahore *et al*.[4] further extended this strategy for synthesis of farinomalein C-E.

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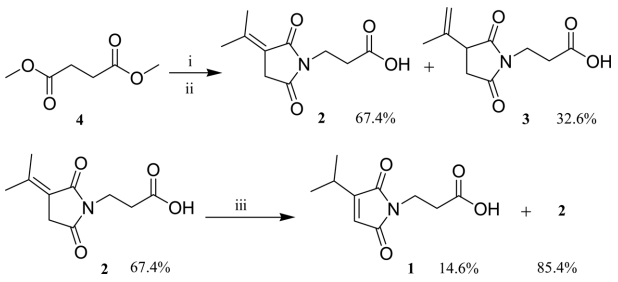
In present work synthesis of farinomalein is attempted using **Scheme 1**. The technique LC-MS/MS facilitate to explore between the isomers and effect of position of the double bond in their structure. The application of LC-MS/MS is also appreciated in structure elucidation of numerous natural products.[5-8]

Experimental

Materials and Methods

The solvents used were distilled and dried as per standard laboratory procedures. Other chemical reagents used were analytical grade with minimum purity 99.5%. The solvent methanol was used for sample preparation unless stated otherwise. Mass spectra were recorded on Agilent® G6540B MS Q-TOF at 70 eV with Electron



Scheme 1. Reagents, conditions and yields: (i) *t*-BuOK, acetone, 15 min, 77%; (ii) beta-alanine, glacial acetic acid, 1.5 h, reflux, 65%; (iii) Ref.[9] 3 steps, 33%.

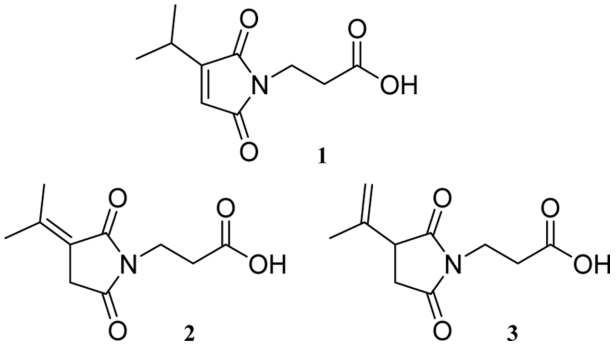


Figure 1. Structure of farinomalein **1**, isomer **2** (*pseudonym*: isofarinomalein) and isomer **3**.

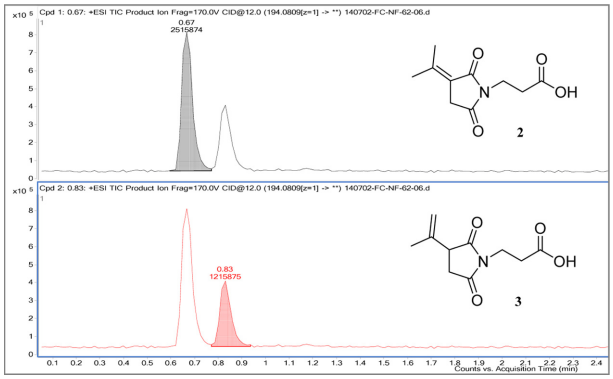


Figure 2. Chromatogram showing peak area for isomers **2** and **3**.

Spray Ionization technique. HPLC grade acetonitrile was obtained from Merck®. De-ionized, pure water was prepared by passing through a Millipore Corp Milli-Q RG purification system. The detailed LC-MS/MS operation method† is available on shared web page.

Results and Discussion

The structures of the farinomalein and its isomers are shown in **Figure 1**. In first attempt, the monoester alkylidenesuccinic acid was achieved in situ by green approach procedure reported in 2009.[10] The reaction carried out with *t*-butoxide, acetone and dimethyl succinate was vigorous and exothermic in nature. The obtained crude product was subjected to condensation with beta-alanine in acetic acid for about hour and half time. This procedure yielded into few milligram of the mixture. The sample was prepared in the spectroscopic grade methanol and subjected to LC-MS/MS analysis. The LC-MS/MS data revealed formation of two isomers in the ratio of 67:33 (**Figure 2**) with identical mass and fragmentation pathway.The compound **2** and **3** are having retention time 0.67 min and 0.83 min respectively.

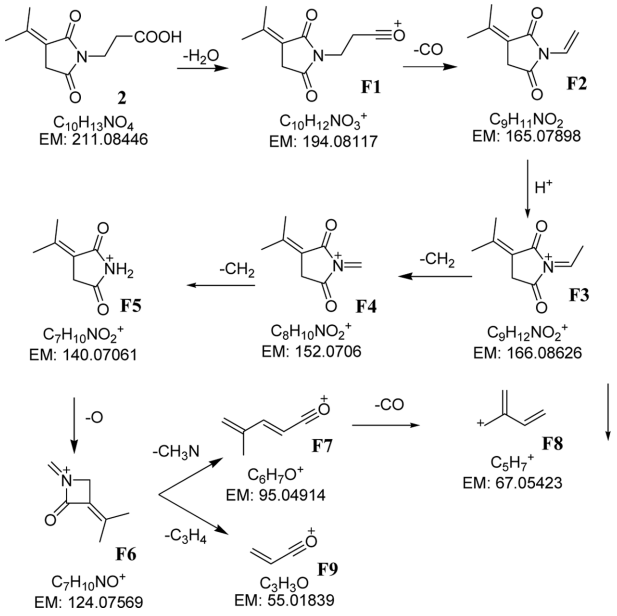


Figure 3. Fragmentation pathway for isomer **2**.

The both chromatograms for isomer **2** and **3** showed **F4** m/z 152.0706 as base peak, whereas molecular ion peaks M^+ or $[M+H]^+$ for the both isomers were not seen.

The molecular ion fragment **F1** was recorded as m/z 194.08117 for isomer **2**. Subsequently, the loss of neutral carbon monoxide led to fragment **F2** m/z 165.07898. The vinyl amide on protonation gave the stable adduct **F3** m/z 166.08626. The consecutive loss of $-CH_2$ formed the fragment **F3** resulting in base peak **F4** and amide fragment **F5** m/z 152.0706 and 140.07061 respectively. The meta-stable fragment **F5** on elemental oxygen loss formed beta-lactum fragment **F6** m/z 124.7659 with unique structural feature and stability.

Further, the elimination of methyl amine moiety formed the moiety formed the fragment **F7**, analiphatic hydrocarbon m/z 95.04914. The fragments **F8** m/z 67.05423 and **F9** m/z 55.01839 were recognized after loss of carbon monoxide and methyl acetylene from the fragment **F7** respectively (**Figure 3**).

The structural assignment to the isomer **3** showed the isomeric fragments **F10**, **F11**, **F12**, **F4**, **F14** and **F15** whereas **F7**, **F8** and **F9** were found identical in fragmentation pathway both the isomers **2** and **3** (**Figure 4**). On magnification of mass spectrogram of isomer **3** on magnification, the moderate and unsigned fragment peak m/z 177.0893 was found. This observation confirmed the presence two isomers in the mixture and overruled the possibility of ghost peak or bleed peak.

Revisiting the literature, it was found that in the year 2000 Tanaka and co-workers[11] studied the Stobbe condensation under solvent free condition, using potassium

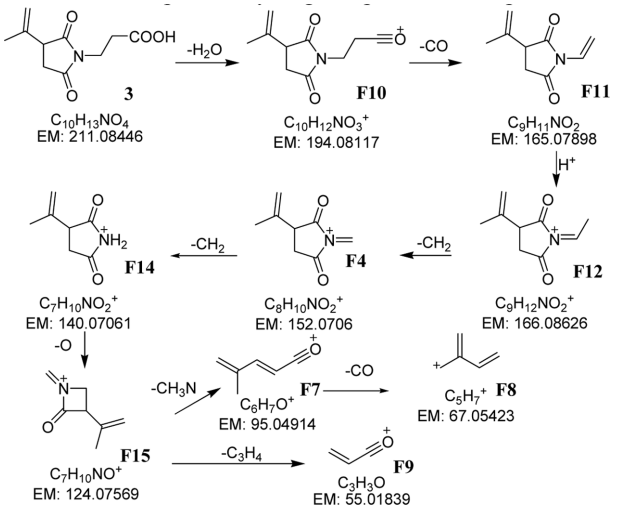


Figure 4. Fragmentation pathway for isomer **3**.

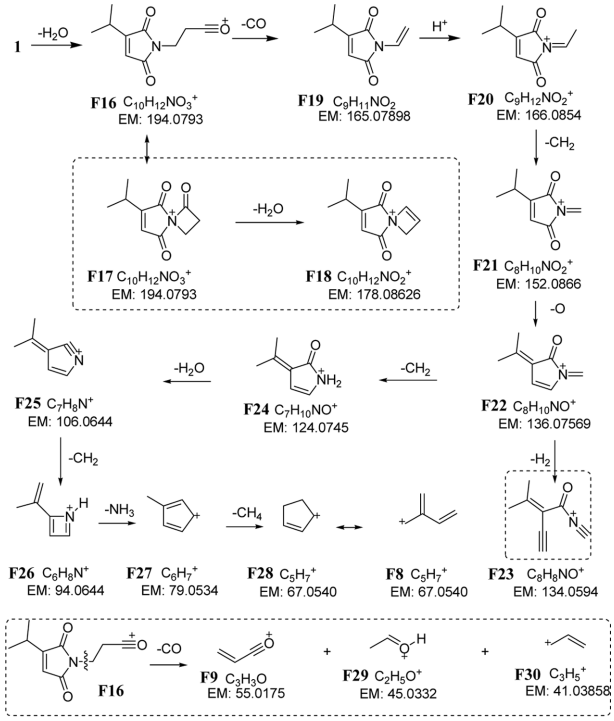


Figure 5. Fragmentation pathway of farinomalein **1.**

tert-butoxide as strong base in the reaction. The occurrence of the isomer **3** in the present scheme was due to formation of kinetically controlled isomer in Stobbe condensation. In order to overcome this problem, another hassle-free procedure was adopted from the literature.[12] The Haval-Argade contrathermodynamic rearrangement sequence was carried on the structurally proven isomer **2** (**Scheme 1**). The two isomers with molecular formula $C_{10}H_{13}NO_4$ were observed in the LC-MS/MS spectrogram.

The farinomalein **1** and isomer **2** were observed with retention time 3.75 min and 5.25 min respectively. Isomer **2** showed the base peak at *m/z* 152.0706, wherein farinomalein[‡] **1** at *m/z* 166.0854. Molecular ion peak M^+ or $[M+H]^+$ for the farinomalein was not seen, since acid has characteristic property of losing water molecule from their structures by ESI. The molecular ion fragment **F16** reported as *m/z* 194.0794. The spiro fragment **F17** is assumed as canonical form for the **F16**, which on water loss led to fragment **F18**. Fragment **F16** subsequently, on loss of neutral carbon monoxide led to fragment **F19** *m/z* 165.07898. This vinyl amide fragment **F19** on protonation gave the stable adduct, base peak the fragment **F20** *m/z* 166.0854. The following losses of methylene, oxygen and methylene were resulted in formation of **F21** m/z 152.0866, **F22** m/z 136.07569 and **F24** m/z 124.0745. The intermediate fragment **F22** on loss of hydrogen molecule opened to fragment **F23** *m/z* 134.0594. The fragment **F24** on loss of water molecule gave fragment **F25** *m/z* 106.0644.

The fragment **F25** on loss of methylene was resulted as **F26** *m/z* 94.0644. The meta-stable **F26** fragment on loss of ammonia led into hydrocarbon skeleton **F27** *m/z* 79.0534.The cyclopetadiene fragment **F27** on methane loss gave two isomeric fragments **F28** and **F8** *m/z* 67.0540. *β*-alanine side chain of farinomalein on fragmentation results in three fragments, those are **F9** (loss of CO), *m/z* 55.0175, **F29** *m/z* 45.0322, **F30** *m/z* 41.03858 (**Figure 5**). The ratio of farinomalein **1** and isomer **2** by ^{1}H NMR and LC-MS/MS was found in coherence with each other.

Conclusions

Mass spectrometry facilitated in the structure determination of farinomalein **1** and its isomer **2** and **3**. The present study provides significant clues for predication of plausible mechanism for fragmentation pathway of isomer **1-3.** The study also repressent an unique characteristic feature of natural product containing alkylmaleimide functional group and also shed some light on effect of position of double bond and fragmentation pathway among isomers of farinomalein.

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Notes and references

[†]Electronic Supplementary Information (ESI) available: [https://drive.google.com/file/d/1ODcVeq26t0YwR9gdjzX

v6y9IOXQqpPCo/view?usp=sharing].

‡Farinomalein **1** is exhibited with following fragment peaks in LC-MS/MS for $C_{10}H_{13}NO_4$ *m/z* (intensity) 194.0793 (37), 178.08626 (3), 166.0854 (100), 152.07061 (34), 134.06004 (20), 124.0745 (23), 106.0644 (76), 94.06513 (53), 79.05423 (69), 73.0540 (15), 67.5423 (20), 55.01784 (32), 45.03349 (22), 41.03858 (12).

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