Original

LC/PAD/APCI-MS for the characterization and analysis of porritoxin and its related compounds from *Alternaria porri.*

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Abstract

LC/PAD/APCI-MS was utilized in the analysis of porritoxin and its structurally related compounds produced by *Alternaria porri.* APCI-MS of the porritoxins produced protonated molecules $[M + H]^+$ and fragment ions attributed to the elimination of side chains. UV spectra provided chromophore information, by which three structural types of porritoxin and its related compounds can be characterized. A combination of APCI-MS and UV spectral data afforded accurate information for the identification of porritoxins by HPLC. This method was utilized in the analysis of porritoxins produced by *A. porri* in five kinds of culture media. Furthermore, this method gave quantitative information about porritoxin, which showed the strongest inhibitory effect on the growth of seedlings of stone leek and lettuce among porritoxins, and displayed excellent linearity from 10 ng/injection to 100 μg/injection. Porritoxin in five kinds of culture media was determined using this method.

Key words: Porritoxin; isoindoline; phthalide; zinniol; Alternaria porri; LC/PAD/APCI-MS

Introduction

Porritoxin (**1**), which was isolated from the culture liquid of *Alternaria porri,* showed an inhibitory effect on the growth of seedlings of stone leek and lettuce [1, 2]. *A. porri,* which is the fungus that causes black spot disease in the stone leek and onion, produces its related compounds, zinnimidine (**2**) [3], 6-(3',3'-dimethylallyloxy)-4-methoxy-5-methylphthalide (**3**) [4], 5-(3',3'-dimethylallyloxy)-7-methoxy-6-methylphthalide (**4**) [3], porritoxinol (**5**) [5], silvaticol (**6**) [6] and zinniol (**7**) [7]. They are classified into the following three categories: i) the isoindoline type (**1** and **2**), ii) the phthalide type (**3**-**6**), and iii) the 1,2-benzenedimethanol type (**7**). Type i compounds have an isoindolinone (benzolactam) chromophore, type ii compounds have a phthalide (benzolactone) chromophore and the type iii compound has a benzene chromophore.

Previously, we reported the determination of **1** (type i), **3**, **5** and **6** (type ii) in the culture liquid of *A. porri* by reverse phase HPLC using UV (254 nm) monitoring [8, 9]. However, fixed wave length UV monitoring detection provides insufficient selectivity and sensitivity for the analysis of porritoxins, because various kinds of compounds are present in the culture liquid. Therefore, a highly selective and sensitive analysis method for all types of porritoxins is required.

LC/MS with atmospheric pressure chemical ionization (APCI) is a powerful technique for the analysis of natural products. Recently, we reported on the performance of positive-ion LC/APCI-MS for the determination of three phytotoxic cyclic tetrapeptides extracted from the culture liquid of *A. porri* [10]. UV spectra provided by a photodiode-array detector (PAD) provided the chromophore information, which cannot be determined from APCI-MS data.

Therefore, we have utilized the combination of PAD and APCI-MS for the rapid and accurate identification of porritoxins by

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HPLC. This paper reports on the performance of LC/PAD/APCI-MS for the rapid and accurate identification of porritoxins and provides an analysis of the productivity of porritoxins produced by *A. porri* in five different culture media.

Experimental

General.

A Hitachi M-1200 quadrupole mass spectrometer was connected to a Hitachi L-2130 liquid chromatograph with a PAD (Hitachi L-2450), a UV detector (Hitachi L-2420), a column oven (Hitachi L-2300) and a system manager (Hitachi EZChrom Elite).

Fungus.

The strain of *Alternaria porri* used in this experiment was purchased from IFO (Institute for Fermentation Osaka), strain number 9762.

*Authentic samples (**1**-**7**).*

The structures of porritoxins (**1**-**7**) used in this study are shown in Formula. They were obtained from the culture liquid of *A. porri,* which was cultured in Richards' medium for 35 days. The isolation procedures and physicochemical properties of these compounds have been previously reported [1-6, 11].

Culture liquid.

A. porri was cultured in Czapek Dox, Czapek Dox with $ZnSO_4$, Raulin Thom, Richards' and Richards' with $(NH_4)_2SO_4$ media for 35 days at 25℃. The compositions of Czapek Dox, Czapek Dox with $ZnSO_4$, Raulin Thom and Richards' media have been described previously [1, 12, 13]. The composition of Richards' with $(NH_4)_2SO_4$ medium was supplemented with $(NH_4)_2SO_4$ (2.5 mg/L) in Richards' medium.

Procedure for LC/PAD/APCI-MS.

After culturing, the 1 L of culture liquid was filtered through gauze. The culture filtrate was extracted with ethyl acetate. The ethyl acetate solution was dried in an evaporator. The 0.5 (w/v) % methanol solution of dry ethyl acetate extract was prepared for HPLC. A sample of 10 μL was injected into the column. The positive-ion APCI mass spectra were acquired by scanning from *m/z* 70 to 600. The APCI interface section was set at a drift voltage of 60 V, a multiplier voltage of 1800 V, a needle voltage of 3000 V and a nebulizer temperature of 200℃. The UV absorption spectra were recorded from 210 to 350 nm using a PAD. The solvent system was 50 % acetonitrile / water for all porritoxins. The column was Wakopak Wakosil-Ⅱ5C18 RS 250 mm L. × 4.6 mm I.D. (Wako). The mobile phase flow rate was 1.0 mL/min at a column temperature of 25℃.

Results and discussion

APCI-MS of porritoxins.

The typical ions resulting from positive ion APCI mass spectra of **1**-**7** obtained by LC/APCI-MS are shown in Table 1. APCI-MS produced protonated molecules $[M + H]^+$ for all porritoxins and fragment ions attributed to the elimination of side chains such as $[M + H - C_5H_9]^+$ for **1**-**4**, $[M + H - C_5H_{11}O_2 - CHO]^+$ for **5** and $[M - C_5H_9O]^+$ for **7**. The protonated molecules were observed as base peaks in **1**-**6** belonging to type i and ii compounds. On the other hand, the APCI-MS of **7**, the compound belonging to type iii, showed $[M - C_5H_9O]^+$ as a base peak and the intensity of $[M + H]^+$ was very weak.

UV spectra of porritoxins.

The UV absorption maxima obtained by on-line PAD-HPLC analysis of **1**-**7** and some typical UV spectra are shown in Table 1 and Figure 1. Type i compounds showed UV absorption maxima at

Table 1. Characteristic Ions Resulting from LC/APCI-MS and UV Absorption Maxima Obtained by PAD of Porritoxins

Compound	Formula	Major ions	Identity	Relative abundance (%)	Absorption maxima (nm)
1	$C_{17}H_{23}O_4N$	306	$[M + H]^+$	100	215, 255, 294
		237	$[M + H - C_5H_9]^+$	25.3	
2	$C_{15}H_{19}O_3N$	262	$[M + H]^+$	100	214, 252, 294
		193	$[M + H - C_5H_9]^+$	32.9	
3	$C_{15}H_{18}O_4$	263	$[M + H]^+$	100	215, 254, 301
		194	$[M + H - C_5H_9]^+$	57.0	
4	$C_{15}H_{18}O_4$	263	$[M + H]^+$	100	215, 258
		194	$[M + H - C_5H_9]^+$	43.2	
5	$C_{15}H_{20}O_6$	297	$[M + H]^+$	100	215, 254, 301
		165	$[M + H - C_5H_{11}O_2 - CHO]^+$	52.8	
6	$C_{10}H_{10}O_4$	195	$[M + H]^+$	100	213, 253, 304
7	$C_{15}H_{22}O_4$	267	$[M + H]^+$	1.5	230, 274
		181	$[M - C_5H_9O]^+$	100	

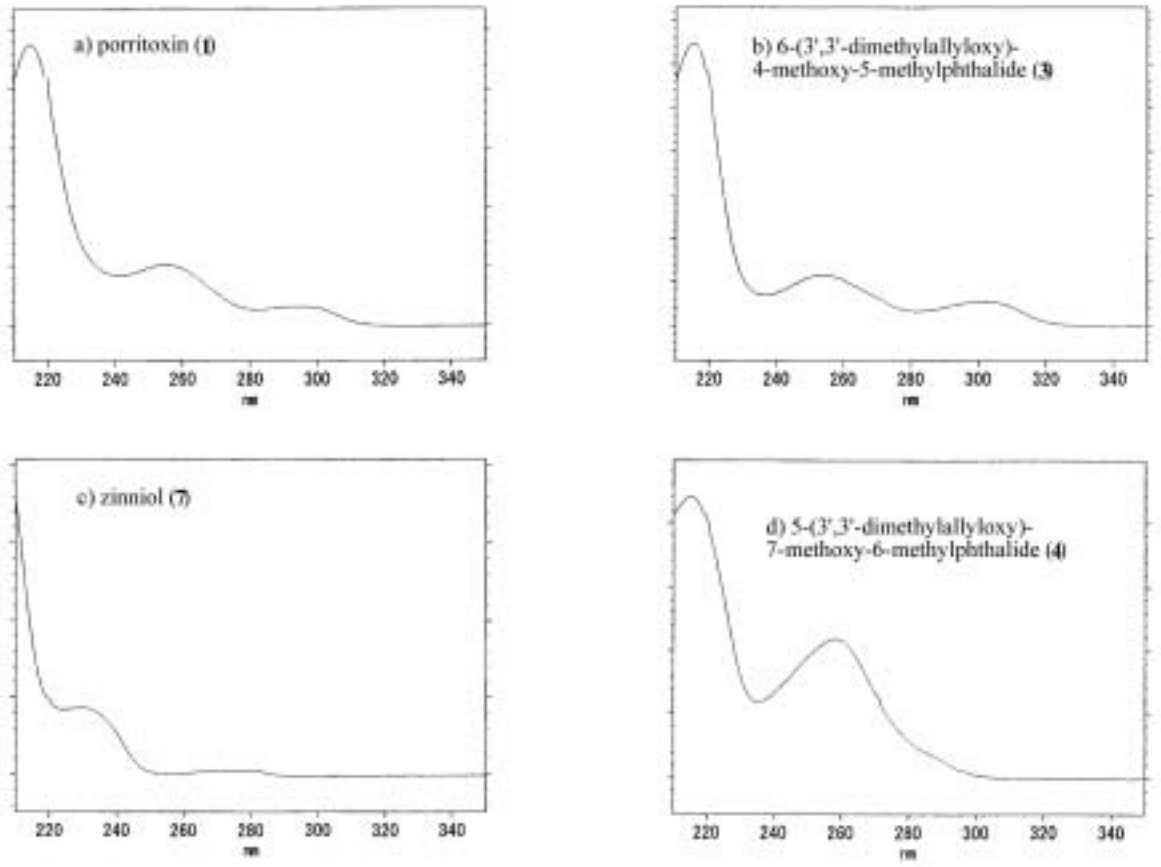
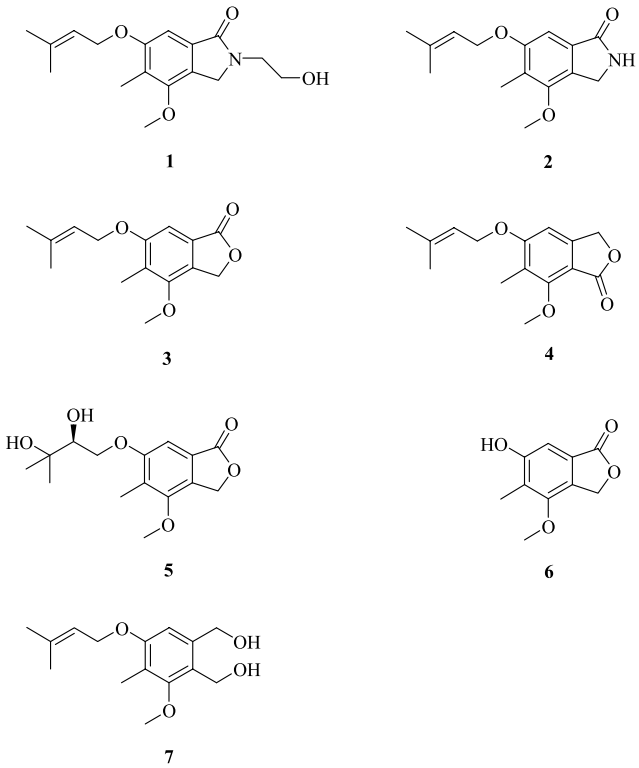


Figure 1. Typical UV spectra of porritoxins.

215, 255 and 294 nm, type ii compounds, except for **4**, at 215, 254 and 301 nm, and the type iii compound at 230 and 274 nm. Therefore, three types of porritoxins could be characterized by UV spectra based on their chromophore system. A slight red shift of the UV maxima in type ii compounds compared with those of type i compounds may reflect the presence of a conjugated lactone or lactam structure. Furthermore, from the UV spectra we can distinguish two structural isomers of phthalides (**3** and **4**) which have the same molecular formula $C_{15}H_{18}O_4$. The structural difference between these two compounds is only in the substituted position of the carbonyl group on the lactone ring. Therefore, APCI-MS could not distinguish them. On the other hand, the UV spectra of both of these compounds exhibited a clear difference as shown in Figure 1. Compound **4** showed a slightly red shifted absorption maxima (λ_{max} 258 nm) compared with **3** (λ_{max} 254 nm) in π-π^* transition. This red shift was assumed to be caused by the difference of the positions of the auxochromes (CH_2, CH_3, OCH_2 and OCH_3) in the benzoyl group and could be explained using Woodward-Fieser rules (calculated absorption maxima of **3** at 257 nm and **4** at 268 nm). An approximately 22 nm blue shift in the absorption maxima in the type iii compound compared with those of type i and ii compounds is due to the lack of the carbonyl group. Consequently, a



Formula

combination of UV and MS spectral data was needed to afford the characterization of all porritoxins by on-line HPLC.

Optimization of HPLC conditions for analysis of all porritoxins in culture medium of A. porri.

The most suitable solvent system using an ODS column for the analysis of all porritoxins was investigated. The influence of the ratio of acetonitrile to water on the separation of porritoxins in the ethyl acetate extract of *A. porri* cultured in Raulin Thom medium is shown in Figure 2. As the ratio of acetonitrile to water increased, the elution times of porritoxins became shorter. In 20 % acetonitrile / water, a good resolution between **5** and **6** was obtained, but the other porritoxins were not eluted within 30 min. On the other hand, 60 % acetonitrile / water eluted all the compounds within 11 min; however, **5** and **6** could not be resolved using this solvent system. 50 % acetonitrile / water eluted all the compounds with sufficient resolution for all porritoxins within 30 min. Therefore, this solvent system was used for the analysis of all porritoxins by HPLC.

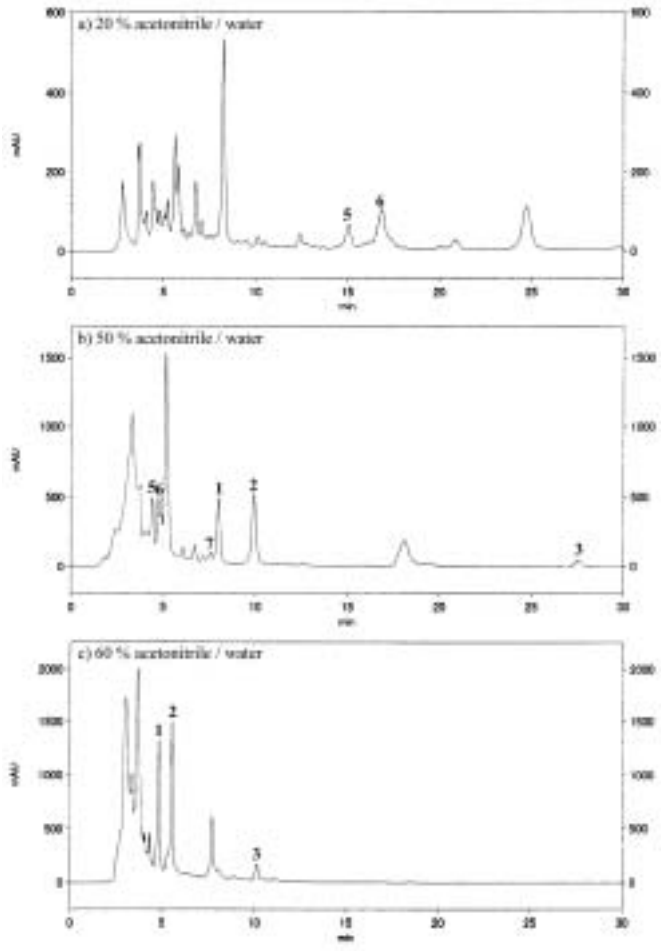


Figure 2. The effect of the ratio of acetonitrile to water on the chromatograms of the ethyl acetate extract from Raulin Thom medium of *A. porri.* HPLC; column: Wakopak Wakosil-II 5C18 250 mm L. × 4.6 mm I. D., flow rate: 1.0 mL/min, mobile phase as listed, chromatograms operating at 215 nm. Peaks 1: porritoxin, 2: zinnimidine, 3:6-(3',3'-dimethylallyloxy)-4-methoxy-5-methylphthalide, 4:5-(3',3'-dimethylallyloxy)-7-methoxy-6-methylphthalide, 5: porritoxinol, 6: silvaticol, 7: zinniol.

Furthermore, **1**, which showed the strongest inhibitory effect on the growth of seedlings of stone leek and lettuce among compounds **1**-**7** [1, 4-6, 14], was determined using this method, because the concentration of **1** was in the quantitative range. A calibration curve that displayed excellent linearity was obtained for **1** from 10 ng/injection to 10 μg/injection; $y = 2.259\times10^{8}$ x ($r^2 = 0.9998$). The quantitative analysis of **1** was performed by on-line PAD-HPLC operating at 215 nm. The concentration of **1** in the five kinds of culture media is shown in Table 2.

Table 2. The Concentration of **1** in Five Different Culture Media

	Concentration (mg/L)				
Compound	Czapek Dox	Czapek Dox with $ZnSO_4$	Raulin Thom	Richards'	Richards' with $(NH_4)_2SO_4$
1	0.876	0.188	0.991	0.296	not detected

Application of LC/PAD/APCI-MS for the analysis of porritoxins in five kinds of culture media of A. porri.

We have applied our combination method for the rapid and accurate evaluation of the productivity of porritoxins in five kinds of culture media of *A. porri.* Porritoxins in the culture liquid were identified on the basis of their retention time, MS spectra and UV spectra provided by LC/PAD/APCI-MS. Raulin Thom medium contains ammonium ions instead of the nitrate ions used in Czapek Dox and Richards' media as a nitrogen source, and contains tartaric acid and $ZnSO_4$. Thus, *A. porri* was cultured in five kinds of culture media: Czapek Dox, Czapek Dox with $ZnSO_4$, Raulin Thom, Richards' and Richards' with $(NH_4)_2SO_4$. The productivity ratio of **1**-**7** in the five kinds of culture media is shown in Table 3.

When *A. porri* was cultured in Raulin Thom medium, which contains ammonium ions as a nitrogen source, isoindolines (**1** and **2**) were produced in the highest yield among the five kinds of culture media. Phthalides (**5** and **6**) except for **3** were also produced in the highest yield in this medium. In the cases where we cultured *A. porri* in Czapek Dox and Richards' media, which contains nitrate ion as a nitrogen source, and Raulin Thom medium, the concentration of **3** was at almost the same level. But the productivities of all porritoxins except for **5** were decreased by the addition of $ZnSO_4$ to Czapek Dox medium. Particularly, those of **3** and **7** were decreased markedly. Moreover, the productivities of all porritoxins were inhibited by the addition of $(NH_4)_2SO_4$ to Richards' medium. These results suggest that not only $(NH_4)_2SO_4$ and $ZnSO_4$ but also tartaric acid and ammonium tartarate in Raulin Thom medium contributed

Table 3. The Productive Ratio of **1**-**7** in Five Different Culture Media

	Relative Productive Ratio (%)				
Compound	Czapek Dox	Czapek Dox with $ZnSO_4$	Raulin Thom	Richards'	Richards' with $(NH_4)_2SO_4$
1	88.4 (59.2)[a]	19.0 (12.7)[a]	100 (66.9)[a]	29.9 (20.0)[a]	0 (0)[a]
2	10.0 (10.0)[a]	4.5 (4.5)[a]	100 (100)[a]	6.2 (6.2)[a]	0 (0)[a]
3	100 (39.9)[a]	4.2 (1.7)[a]	58.3 (23.2)[a]	69.8 (27.8)[a]	0 (0)[a]
4	trace (trace)[a]	0 (0)[a]	trace (trace)[a]	trace (trace)[a]	0 (0)[a]
5	31.7 (3.9)[a]	36.1 (4.4)[a]	100 (12.2)[a]	10.8 (1.3)[a]	0 (0)[a]
6	19.0 (5.0)[a]	8.3 (2.2)[a]	100 (26.5)[a]	6.5 (1.7)[a]	0 (0)[a]
7	100	0	2.5	0.7	0

Values represent percentages relative to the greatest peak area by HPLC in each compound.

[a] Values in parentheses represent percentages relative to the peak area of compound **2** cultured in Raulin Thom medium, the greatest peak area by HPLC in compounds **1**-**6**. Structures of **1**-**6** contain the benzoyl chromophore.

to the productivity of isoindolines and phthalides. On the other hand, the productivity of **7** was also inhibited by the addition of $(NH_4)_2SO_4$, and $ZnSO_4$, but **7** was produced in the highest yield in Czapek Dox medium.

Isoindolines and phthalides (**3**, **5** and **6**) were produced in good yield in Raulin Thom medium. Zinniol (**7**) was produced in good yield in Czapek Dox medium. It was interesting that the most suitable medium for the production of isoindolines and phthalides is different from that of the zinniol-type compound. Although some zinniol related compounds were isolated from *Alternaria solani and A. tagetica* [15-17], we did not isolate them from *A. porri.* The reason may be that *A. porri* was not cultured in Czapek Dox medium, but rather, was cultured in Richards' medium.

In conclusion, LC/PAD/APCI-MS provided a selective and sensitive method for the analysis of porritoxins in the culture liquid of *A. porri.*

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