3-Phenyl-2,7-naphthyridin-1(2H)-one as a New Fluorogenic Reagent for Epoxides and Its Application to Determination of Trichothecene Mycotoxins

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A fluorescence reaction of epoxides with 3-phenyl-2,7-naphthyridin-1(2H)-one (PNO) is described. Common epoxides and 12,13-epoxytrichothecenes gave a green fluorescence (λ_{ex} 441–467 nm and λ_{em} 506–510 nm) after reaction with PNO at 120°C for 90 min in 1,2-ethanediol/2-methoxyethanol mixture for the former and at 150°C for 30 min on a solid phase for the latter, respectively, and followed by addition of *N,N*-dimethylformamide in both the cases. By the proposed methods, as little as 20–30 pmol of common epoxides and 64–85 pmol of trichothecenes could be determined with relative standard deviations of ranges 1.7–4.3% and 4.8–8.6%, respectively.

Keywords Epoxides, trichothecene mycotoxins, alkylating agents, fluorescence reaction, 3-phenyl-2,7-naphthyridin-1(2H)-one

Trichothecene mycotoxins, having a characteristic 12,13-epoxytrichothec-9-ene structure, are naturally occurring fungal toxic sesquiterpenoids produced by various species such as *Fusarium*, *Myrothecium*, *Trichothecium* and *Cephalosporium*. There have been many reports that these toxins cause a number of illnesses in man and animals, and trichothecenes are also found in foods and feeds in Japan, United States of America, Canada, England and several other countries.^{1,2} Thus various following methods have been developed for determining trichothecenes: gas chromatography^{3–5}, thin-layer chromatography^{6–8}, high performance liquid chromatography^{9,10}, polarography¹¹ and immunoassay.^{12,13} Gas chromatography is highly sensitive and is the technique most used for deter-

mination of trichothecenes. But erroneous identification can sometimes occur^{14,15}, although, the use of expensive mass spectrometer as a detector can avoid this problem.

Sub-nanomol amounts of some epoxides could be determined fluorometrically by their reaction with nicotinamide and active methylenes (NA method)^{16,17}, and it has been confirmed that the fluorophore has a 2,7-naphthyridine structure.^{17,18} And the NA method has been applied to the fluorodensitometric determination of trichothecenes.⁷

Although, in the NA method sensitivity and fluorophore stability were satisfactory, the experimental procedures were rather complicated because of the necessity of multi-reaction steps. If 3-phenyl-2,7-naphthyridin-1(2H)-one (PNO), which corresponds to the de-alkylated derivative of the fluorophore resulted by the NA method, is used as a reagent, epoxides are expected to give the same reaction product with the NA method by only one step (Fig. 1). Therefore, under the such consideration, the present study aimed at establishing a simple fluorometric method (PNO method) for determining common epoxides with PNO and then, we attempted to apply the PNO method for determining trichothecene mycotoxins.

Fig. 1 Reaction of epoxides with nicotinamide (a) and PNO (b).

Experimental

Preparation of PNO

PNO was prepared according to the method of

Kröhnke *et al.*¹⁹ The product was further purified by repeated recrystallization from ethanol as white needles: mp. 234.5–235.5°C (uncorrected; 237–239°C¹⁹, 239–239.5°C²⁰). Anal. Calcd for C₁₄H₁₀N₂O: C, 75.70; H, 4.54; N, 12.61. Found: C, 75.70; H, 4.61; N, 12.56. Although Kröhnke *et al.* reported that PNO had a 3-phenyl-2,7-naphthyridin-1(4*H*)-one structure, this structure has been corrected to 3-phenyl-2,7-naphthyridin-1(2*H*)-one by Bobbitt *et al.*²⁰

Chemicals

PNO was used as a 0.5% (w/v) solution in 1,2-ethanediol/2-methoxyethanol (1:1) and a 0.1% (w/v) solution in ethanol. Stock standard solutions of common epoxides, alkyl halides (Tokyo Kasei Co.), *O*-*p*-nitrophenyl phosphorothioate (parathion, purchased from Wako Pure Chemical Industry Ltd.), *p*-[bis-(2-chloroethyl)amino]-L-phenylalanine (melphalan), 1,6-dibromo-1,6-dideoxy-D-mannitol (mitobronitol, purchased from Sigma Chemical Co.), 1,4-butanediol dimethanesulfonate (busulfan, supplied by Takeda Pharmaceutical Co.), 2-[bis-(2-chloroethyl)amino] tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (cyclophosphamide, supplied by Shionogi Pharmaceutical Co.), 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride (nimustine, supplied by Sankyo Pharmaceutical Co.) and tris-(1-aziridinyl)phosphine sulfide (thiotepa, supplied by Sumitomo Chemical Co.) were prepared at 2–10 mM concentrations in 2-methoxyethanol.

The trichothecene mycotoxins used in this study, 4*β*-

hydroxy-3*α*,15-diacetoxy-8*α*-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene (T-2 toxin), 3*α*-hydroxy-4*β*,15-diacetoxy-12,13-epoxytrichothec-9-ene (diacetoxyscirpenol), 3*α*,7*α*,15-trihydroxy-4*β*-acetoxy-12,13-epoxytrichothec-9-en-8-one (fusarenone-X), 3*α*,7*α*,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol) and 3*α*,4*β*,7*α*,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one (nivalenol) are purchased from Wako Pure Chemical Industry Ltd. These structures were listed in Table 1. Stock standard solutions of trichothecenes were prepared at 1–2 mM concentrations in methanol.

Except for the use for PNO preparation, other chemicals used were of analytical-reagent grade.

Apparatus

A Shimadzu RF-502 spectrofluorometer equipped with a xenon lamp was used with a 4×10 mm quartz cell at room temperature. The spectral bandwidths were 5 nm (excitation) and 4 nm (emission). All fluorescence excitation and emission spectra are uncorrected. A dry block heater (DRI-BLOCK DB-3H, M&S Instruments) was used for the reaction.

Procedure for determination of common epoxides

To a 10 µl of sample solution in a 1.5-ml glass-stoppered test tube, add 100 µl of the 0.5% PNO solution, and place the tube in a block heater at 120°C for 90 min. After cooling it to room temperature, dilute the solution with 500 µl of *N,N*-dimethylformamide (DMF) and measure the fluorescence intensity at respective excitation and emission wavelengths.

Procedure for determination of trichothecene mycotoxins

Pipette a 10 µl of sample solution in 1.5-ml glass-stoppered test tube and evaporate to dryness. Add to it 20 µl of the 0.1% PNO solution and evaporate to dryness again. Place the tube in a block heater at 150°C for 30 min. After cooling it to room temperature, add 600 µl of DMF to the mixture and measure the fluorescence intensity with excitation at 467 nm and emission at 507 nm.

Table 1 Structures of trichothecene mycotoxins

	R ₁	R ₂	R ₃	R ₅
T-2 toxin	OH	OCOCH ₃	OCOCH ₃	OCOCH ₂ CH(CH ₃) ₂
Diacetoxy-scirpenol	OH	OCOCH ₃	OCOCH ₃	H
	R ₁	R ₂	R ₃	R ₄
Fusarenone-X	OH	OCOCH ₃	OH	OH
Deoxynivalenol	OH	H	OH	OH
Nivalenol	OH	OH	OH	OH

Results and Discussion

Determination of common epoxides

Experimental conditions were examined by using 1,2-epoxy-3-phenoxypropane (EPP) as a representative sample of common epoxides.

2-Methoxyethanol and 1,2-ethanediol/2-methoxyethanol mixed solvent were selected for sample and PNO, respectively, because of their excellent features with regards to non-volatility, solubility of PNO and the fluorescence intensity of the resulting products. A ratio of 1:1 was selected as the optimal value for the 1,2-ethanediol/2-methoxyethanol mixture.

The reaction product of EPP showed a blue fluorescence (excitation at 392 nm and emission at 434

Table 2 Effect of dilution^a

Solvent	$\lambda_{\max}/\text{nm}$		RFI ^b	Test/ blank
	Excita- tion	Emis- sion		
No dilution	392	434	515	0.8
<i>N,N</i> -dimethyl- formamide (DMF)	451	507	100	66
Ethanol	428	500	91	33
Dioxane	421	490	90	40
2-Methoxyethanol	428	500	87	31
Dimethylsulfoxide	451	507	80	46
Acetone	435	506	35	28

a. EPP (1 nmol) was reacted with the same manner as described in the standard procedure, and then diluted with 500 μl of each solvent.

b. Relative fluorescence intensity; the intensity in DMF is arbitrarily taken as 100.

Fig. 2 Fluorescence excitation and emission spectra of the reaction mixture of EPP and the reagent blank obtained by the standard procedure, and the isolated fluorophore in the NA method.¹⁷ (a), Excitation spectra; (b), emission spectra. Reaction mixture obtained from 1 nmol of EPP (—); the reagent blank (---); 1 nmol of the fluorophore dissolved in the reagent blank solution (---).

nm), while PNO itself showed a blue-purple fluorescence (excitation at 384 nm and emission at 426 nm). But when DMF was used as a diluent, the fluorescence maximum of the reaction product shifted to excitation at *ca.* 450 nm and emission at *ca.* 500 nm, while PNO itself was not affected. DMF was chosen as a diluent because it gave a lower background signal as well as a higher fluorescence intensity of the fluorophore (Table 2), and also because of the stability of the fluorophore. The fluorescence was stable at least 2 h at room

Fig. 3 Effect of reaction temperature and time on the reaction of deoxynivalenol (1.7 nmol) with PNO: (■) 130°C; (□) 140°C; (●) 150°C; (○) 160°C.

temperature.

The effect of PNO concentration on the fluorescence intensity was examined. The fluorescence intensity was increased from 0.1 to 0.3%, then slowly up to the saturated concentration (*ca.* 0.55%). A 0.5% solution was used in the present study.

The relationship between fluorescence intensity and reaction time was studied in the temperature range 100–120°C. Almost constant intensity was obtained after heating at 120°C for 80 min, but in the temperature below 110°C, the reaction was incomplete even if after 2 h. Thus reaction at 120°C for 90 min was selected.

The reaction product of EPP obtained by the proposed method and the isolated fluorophore in the NA method¹⁷ were submitted to silica gel thin-layer chromatography¹⁷ [solvent system: acetone-methanol-formic acid (17:2:1)]; every blue fluorescent spot showed the same R_f value (R_f 0.33). The fluorescence spectra of the isolated fluorophore in the NA method dissolved in a blank solution obtained by the present method was also identical with those obtained by the reaction of EPP with PNO (Fig. 2). Thus, it could be confirmed that the same naphthyridine derivative was produced in both the methods. The reaction yield of EPP could be estimated to be 74% by comparison with both of the fluorescence intensities.

The fluorescence characteristics and their intensities of common epoxides and other some alkylating agents such as alkyl halides, antitumour agents and organophosphate are given in Table 3.

Linear calibration curves were obtained over the concentration ranges of 0.02–2 nmol/10 μl for EPP and 0.03–3 nmol/10 μl for 1,2-(epoxyethyl)benzene (EEB). The relative standard deviations (R.S.D., $n=10$) were 3.7% at 0.05 nmol and 1.7% at 1 nmol for EPP and 4.3% at 0.08 nmol and 2.6% at 2 nmol for EEB. When the NA method¹⁷ was carried out the same sample volume with the PNO method, the reproduc-

Table 3 Relative fluorescence intensities of epoxides and other alkylating agents

Compound ^a	$\lambda_{\max}/\text{nm}$		RFI ^b
	Excitation	Emission	
1,2-Epoxyoctane	441	506	17
1,2-Epoxydecane	441	506	28
1,2-Epoxytetradecane	441	506	42
1,2,3,4-Diepoxybutane	451	508	124
1,2-(Epoxyethyl)benzene (EEB)	451	507	50
1,2-Epoxy-3-chloropropane	451	510	40
1,2-Epoxy-3,3,3-trichloropropane	451	508	68
1,2-Epoxy-3-phenoxypropane (EPP)	451	507	100
1,2-Epoxy-3-butoxypropane	451	507	49
1,2-Epoxy-3-methoxypropane	451	507	30
1,2-Epoxy cyclohexane	442	506	3.1
1,2-Epoxy cyclododecane	442	509	0.2
1-Chlorohexane	442	508	0.3
1-Bromohexane	441	508	4.9
1-Iodohexane	441	508	11
Busulfan	440	508	59
Cyclophosphamide	440	507	33
Melphalan	450	511	27
Mitobronitol	440	506	82
Nimustine	440	507	9.1
Thiotepa	440	507	72
Parathion	440	507	6.0

a. Each compound (2 nmol) was treated in the same manner as in the standard procedure.

b. Relative fluorescence intensity; the intensity from EPP is arbitrarily taken as 100.

Table 4 Relative fluorescence intensities of trichothecene mycotoxins

Compound ^a	$\lambda_{\max}/\text{nm}$		RFI ^b
	Excitation	Emission	
T-2 toxin	467	507	178
Diacetoxyscirpenol	467	508	140
Fusarenone-X	467	506	122
Deoxynivalenol	467	507	100
Nivalenol	467	507	119

a. Each compound (0.43–0.68 nmol) was treated in the same manner as in the standard procedure.

b. Relative fluorescence intensity; the intensity from deoxynivalenol is arbitrarily taken as 100.

ibilities were inferior to the PNO method. Therefore, PNO seems to be useful reagent for the fluorometric analysis of common epoxides.

Determination of trichothecene mycotoxins

In preliminary experiments, it was found that the direct application of the above method to the determination of trichothecenes was difficult because of less reactivity of these compounds (below 1% of the fluorescence intensity of EPP). Therefore, we tried a solid phase reaction. The reaction conditions were examined by using deoxynivalenol because it was most

important one among trichothecene mycotoxins.

When the reaction of deoxynivalenol with PNO was carried out with evaporation after mixing with sample and reagent, reproducibilities of the results were unsatisfactory. The each of evaporation steps was necessary. Changes in the concentration of the PNO solution from 0.03–0.15% had no effect on the fluorescence intensity; therefore, the 0.1% solution was chosen.

The relationship between fluorescence intensity and reaction time was studied in the temperature range 130–160°C. The best result was obtained when reaction was done at 150°C and constant fluorescence intensity was obtained after 30–50 min (Fig. 3), 30 min being convenient.

The fluorescence characteristics and their intensities of trichothecenes are summarized in Table 4.

The calibration curves were constructed for deoxynivalenol and nivalenol. The fluorescence intensities were proportional to the sample concentration in the range 0.085–1.7 nmol/10 μl (25–500 ng/10 μl) for the former and 0.064–0.64 nmol/10 μl (20–200 ng/10 μl) for the latter. The R. S. D. ($n=10$) were 4.8% at 0.17 nmol and 5.8% at 1.4 nmol for deoxynivalenol and 8.6% at 0.16 nmol and 6.1% at 0.64 nmol for nivalenol.

Finally, our method was applied to the determination of deoxynivalenol in spiked corn samples. The clean-up method of Visconti *et al.*¹¹ was used with slight modification in several points: re-extraction of deoxynivalenol from aqueous methanol was performed by ethyl acetate instead of chloroform; preparative thin-layer chromatography was carried out by using 10×20 cm pre-coated TLC plates silica gel F₂₅₄ with concentration zone (0.25 mm thickness, E. Merck) and with a solvent system chloroform–acetone–2-propanol (8:1:1).⁸ The toxin band (R_f 0.43–0.54) was scraped off. The original corn was treated in the same manner and the fluorescence intensity resulted from the band corresponding to deoxynivalenol was used as a blank value. Deoxynivalenol was determined according to the standard addition method. The recovery in the method was found to be 54±5% ($n=3$) at deoxynivalenol concentration level of 1 ppm. Detection limit of deoxynivalenol for overall procedure was about 0.4 ppm for a sample size 50 g; the higher detection limit resulted by the higher background fluorescence. But in preliminary examination, an additional clean-up step by using Sep-Pak florisil cartridges (Waters Associates) or cyano extraction column (J. T. Baker Chemical Co.)²¹ was effective to eliminate the interfering substances. Further studies of clean-up procedure are in progress as the next study.

References

1. Y. Ueno, Ed., "Developments in Food Science 4; Trichothecenes—Chemical, Biochemical and Toxicological

- Aspects*", Kodansha, Tokyo/Elsevier, Amsterdam (1983).
2. H. Kurata and Y. Ueno, Eds., "Developments in Food Science 7; Toxigenic Fungi—Their Toxins and Health Hazard", Kodansha, Tokyo/Elsevier, Amsterdam (1984).
 3. H. Kamimura, M. Nishijima, K. Yasuda, K. Saito, A. Ibe, T. Nagayama, H. Ushiyama and Y. Naoi, *J. Assoc. Off. Anal. Chem.*, **64**, 1067 (1981).
 4. P. M. Scott, P.-Y. Lau and S. R. Kanhere, *J. Assoc. Off. Anal. Chem.*, **64**, 1364 (1981).
 5. T. Tanaka, A. Hasegawa, Y. Matsuki, K. Ishii and Y. Ueno, *Food Addit. Contam.*, **2**, 125 (1985).
 6. S. Takitani, Y. Asabe, T. Kato, M. Suzuki and Y. Ueno, *J. Chromatogr.*, **172**, 335 (1979).
 7. A. Sano, Y. Asabe, S. Takitani and Y. Ueno, *J. Chromatogr.*, **235**, 257 (1982).
 8. M. W. Trucksess, S. Nesheim and R. M. Eppley, *J. Assoc. Off. Anal. Chem.*, **67**, 40 (1984).
 9. A. Visconti and A. Bottalico, *Chromatographia*, **17**, 97 (1983).
 10. H. L. Chang, J. W. DeVries, P. A. Larson and H. H. Patel, *J. Assoc. Off. Anal. Chem.*, **67**, 52 (1984).
 11. A. Visconti, A. Bottalico, F. Palmisano and P. G. Zambonin, *Anal. Chim. Acta*, **159**, 111 (1984).
 12. S. Lee and F. S. Chu, *J. Assoc. Off. Anal. Chem.*, **64**, 156 (1981).
 13. H. Peters, M. P. Dierich and K. Dose, *Hoppe-Seyler's Z. Physiol. Chem.*, **363**, 1437 (1982).
 14. C. J. Mirocha, S. V. Pathre and J. Behrens, *J. Assoc. Off. Anal. Chem.*, **59**, 221 (1979).
 15. A. Visconti and F. Palmisano, *J. Chromatogr.*, **252**, 305 (1982).
 16. H. J. C. F. Nelis and J. E. Sinsheimer, *Anal. Biochem.*, **115**, 151 (1981).
 17. A. Sano, Y. Asabe, M. Suzuki and S. Takitani, *Bunseki Kagaku*, **32**, E93 (1983).
 18. A. Sano, Y. Asabe and S. Takitani, *Bunseki Kagaku*, **32**, E371 (1983).
 19. F. Kröhnke, K. Ellegast and E. Bertram, *Liebigs Ann. Chem.*, **600**, 198 (1956).
 20. J. M. Bobbitt and R. E. Doolittle, *J. Org. Chem.*, **29**, 2298 (1964).
 21. H. Cohen and M. Lapointe, *J. Assoc. Off. Anal. Chem.*, **65**, 1429 (1982).

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