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Short Communication

Studies on stress metabolites

XVI☆. High-performance liquid chromatographic analysis of cruciferous phytoalexins using complex ternary mobile phase gradients

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ABSTRACT

A reversed-phase high-performance liquid chromatographic analysis of cruciferous phytoalexins, antimicrobial compounds synthesized *de novo* by plants after exposure to microorganisms, was performed using an acetonitrile–methanol–water complex solvent gradient system. A well resolved chromatogram of thirteen phytoalexins and three related indole metabolites isolated from cruciferous plants was obtained by this method. Accumulation of phytoalexins in *Pseudomonas cichorii*-inoculated turnip tissue was followed by this high-performance liquid chromatographic analysis.

INTRODUCTION

Phytoalexins are low-molecular-weight antimicrobial compounds that are synthesized *de novo* by plants in response to infection by microorganisms [for recent reviews see refs. 1–3). Accumulation of phytoalexins is considered to be one of the important disease resistance mechanisms. We have recently isolated and characterized several phytoalexins (**1–12**) (Fig. 1) from cruciferous plants, Japanese radish (*Raphanus sativus*) [4], Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) [5–7], cabbage (*B. oleracea*) [8–10] and turnip (*B. campestris* L. ssp *rapa*) [11].

These phytoalexins are structurally unique indole or indole-related compounds possessing one or two sulphur atoms. Devys and co-workers [12,13] have described the accumulation of additional phytoalexins, brassilexin (**13**) and cyclobrassinin sulphoxide, in Indian mustard (*B. juncea*) treated with abiotic elicitors. Also, Dahiya and Rimmer [14] have reported the isolation of methoxybrassinin (**2**) and cyclobrassinin (**4**) from oilseed rape (*B. napus*) inoculated with *Leptosphaeria maculans*. Phytoalexin accumulation was related to resistance to *L. maculans*, a fungus which causes the blackleg disease of crucifers [15,16].

Cruciferous plants contain a group of structur-

☆ For part XV, see ref. 10.

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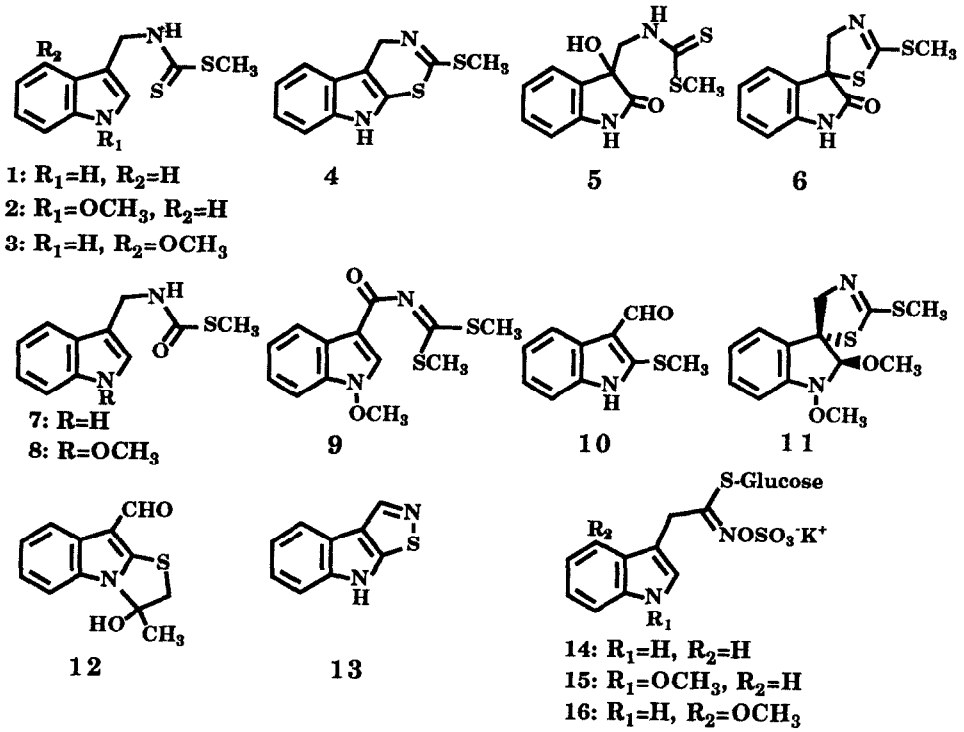

Fig. 1. Chemical structures of cruciferous phytoalexins and indole glucosinolates. **1** = Brassinin; **2** = methoxybrassinin; **3** = 4-methoxybrassinin; **4** = cyclobrassinin; **5** = dioxibrassinin; **6** = spirobrassinin; **7** = brassitin; **8** = methoxybrassitin; **9** = methoxybrassenin B; **10** = brassicanal A; **12** = brassicanal B; **13** = brassilexin; **14** = glucobrassicin; **15** = neoglucobrassicin; **16** = 4-methoxyglucobrassicin. Compound **11** was isolated from both Chinese cabbage and cabbage, but its structure has not been published. The absolute configurations of **6** and **11** have not been determined as yet. The spectroscopic data will be published elsewhere.

ally related secondary metabolites, the glucosinolates (**14**–**16**) [17]. The breakdown of such compounds, brought about by the action of another enzyme, thioglucoside glucohydrolase, yields a variety of products possessing a variety of biological effects, especially indole glucosinolates [18,19]. Recently, interest in the biosynthetic relationship between these indole or indole-related phytoalexins and indole glucosinolates has increased.

In order to study the biosynthesis of the cruciferous phytoalexins, it is important to develop an efficient method of analysing all phytoalexins and related metabolites. In this paper, we describe a practical high-performance liquid chromatographic (HPLC) analysis of these thirteen cruciferous phytoalexins and three related indole metabolites in a single run. We believe that this method is very useful and powerful for research on the biosynthesis of cruciferous phytoalexins and the analysis of phytoalexins of other crucifers.

EXPERIMENTAL

Apparatus

The HPLC analyses were performed on a Japan Spectroscopic (Tokyo, Japan) liquid chromatographic system equipped with a Model 801-SC system controller, a Model 851-AS automatic sampler, a Model 880-50 line degasser, a Model 880-02 low-pressure gradient unit, a Model 865-CO column oven, a Model 880-PU pump and a Model UVI-DEC-100-V UV detector. A Chromatopac C-R6A integrator was used for measuring peak areas (Shimadzu, Tokyo, Japan).

HPLC analytical conditions

The analytical column was μBondapak C_{18} (10 μm, irregular) (300 mm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, USA). Water, methanol and acetonitrile used as the mobile phase were HPLC grade (Wako Chemicals, Tokyo, Japan, and Kanto

Chemicals, Tokyo, Japan). These solvents were kept at 35°C in a water bath. The HPLC column was eluted with water (A)–methanol (B)–acetonitrile (C) under a ternary gradient mode [20–22] at a flow-rate of 2 ml/min at 35°C in a column oven. The column was eluted for 4 min with 75% A + 10% B + 15% C, followed by successive linear gradients: to 60% A + 10% B + 30% C over 10 min; to 50% A + 50% B + 0% C over 3 min; to 100% B over 15 min. The eluent was held at 100% B for 5 min and then brought back to 75% A + 10% B + 15% C over 5 min, and allowed to equilibrate for 5 min (Fig. 2). The UV detector was operated at 254 nm.

Phytoalexin standards

An authentic standard mixture was prepared from thirteen phytoalexins (Fig. 1, **1–13**) and three related indole compounds [3-indolecarboxaldehyde (**17**), 3-indolylacetonitrile (**18**) and 1-methoxy-3-indolecarboxaldehyde (**19**)]. These were isolated from *Pseudomonas cichorii*-inoculated Japanese radish [4], Chinese cabbage [5–7] and cabbage [8–10] as previously described, except for brassilexin (**13**) [12]. Authentic brassilexin (**13**) was supplied by Dr. Barbier (Institut de Chimie des Substances Naturelles, CNRS, Gif-sur-Yvette, France). The authentic standard mixture contained 0.1 μg of each component in 5 μl of methanol. Cyclobrassinin sulphoxide was not determined in this analysis. The internal standard wighteone (**20**) was isolated from *Ficus carica* inoculated with *Fusarium solani* [23].

Quantitative analysis of phytoalexins

Under the analysis conditions, the retention times (in minutes) of the phytoalexins were as follows: 3-indolecarboxaldehyde (**17**), 6.6 ± 0.2; dioxibrassinin (**5**), 9.6 ± 0.2; 3-indolylacetonitrile (**18**), 11.4 ± 0.3; brassicanal B (**12**), 12.0 ± 0.2; brassitin (**7**) and brassicanal A (**10**), 12.6 ± 0.2; 1-methoxy-3-indolecarboxaldehyde (**19**) and brassilexin (**13**), 13.3 ± 0.2; spirobrassinin (**6**), 15.5 ± 0.2; methoxybrassitin (**8**), 18.7 ± 0.3; brassinin (**1**), 19.9 ± 0.3; 4-methoxybrassinin (**3**), 21.3 ± 0.3; methoxybrassinin (**2**), 24.4 ± 0.2; cyclobrassinin (**4**), 24.9 ± 0.2; methoxybrassenin B (**9**), 25.3 ± 0.1; and compound **11**, 26.7 ± 0.2 min. Each value is the mean ± S.D.

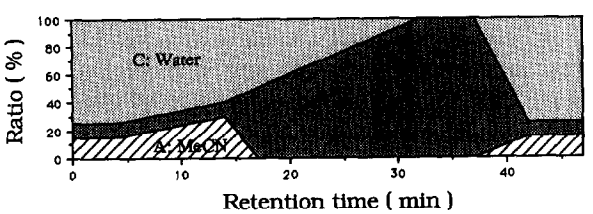

Fig. 2. Solvent composition in the gradient system.

A known quantity (0.50 μg) of wighteone (**20**) was chromatographed as an internal standard. The area of each peak in comparison with that of the standard was used for quantitative purposes. In the case of brassinin (**1**), the calculated equation for the regression curve at 254 nm was $y = -0.002 + 0.229\,x$ where y = weight in μg and x = ratio of peak area between brassinin (**1**) and wighteone (**20**) with $r = 0.997$. The regression line was linear from 0.05 to 2.5 μg. The other areas were calculated from their absorption coefficients recorded with a Japan Spectroscopic Ubest-30 spectrophotometer. The UV detector permitted detection of 10 ng of the substances. Statistical analyses were obtained through Delta Graph 1.5 software, loaded onto an Apple Macintosh SE computer (Apple Computer, Cupertino, CA, USA).

Material

Turnip plants (*B. campestris* L. ssp. *rapa* cv. Tokyo Cross) were cut into 3-mm-thick discs (*ca.* 5 cm in diameter). After an ageing period of 24 h, the discs were divided into two series: control tissues and tissues inoculated with *P. cichorri*. The control discs were incubated at 25 °C for the indicated periods. Two discs were taken out each time and freeze dried. The other series of discs were inoculated with *P. cichorii* (*ca.* 10^8 cells/ml). The inoculated discs and controls were kept under similar conditions and processed similarly. Freeze-dried disc tissue was suspended in acetone and homogenized using a Polytron homogenizer (5 min); after being set aside for 5 min the mixture was filtered under vacuum. The filtrate was evaporated to dryness under reduced pressure and taken up in methanol (2 × 2 ml). The methanol solution was passed through Adsorbex SI (100 mg) and Adsorbex RP-18 (100 mg) cartridges connected in a series, to remove highly polar/non-polar substances, and the cartridges were washed with 5 ml of methanol. The combined methanol eluate and washings were evaporated under vacuum and the residue redissolved in a proportional amount of methanol [11].

RESULTS AND DISCUSSION

An authentic standard mixture was prepared from the thirteen phytoalexins and three related indole metabolites and their resolution was investigated using HPLC under several analytical conditions. At first, two simple gradient systems, acetonitrile–water and methanol–water, were tried. However, a well resolved chromatogram could not be obtained. In the case of the acetonitrile–water gradient system, peaks of polar compounds were well resolved, but those of non-polar compounds were not. On the other hand, in the case of the methanol–water gradient system, the opposite result was obtained. So an acetonitrile–methanol–water combination [20–22] was adopted, and gradient conditions were examined. Several kinds of columns were also tried, *e.g.* RCM8 × 10 with cartridge columns of 10-μm Radial-PAK 5PAH (C_{18}), 10-μm 8MBPH (alkylphenyl type), 10-μm 8CN and μBondapak C_{18} (Waters). When the 10-μm 5PAH cartridge column was used, eight compounds were eluted within 10 min without separation, and all peaks were broadened considerably. In the case of 10-μm 8BPH, most peaks were symmetrical, but only eleven peaks were observed. Sharp peaks could be obtained with 10-μm 8CN columns, but all phytoalexins were eluted within 17 min without complete separation because of weak affinity between the CN group and the indole phytoalexins. Finally, the best resolution was found with the μBondapak C_{18} column using the complex solvent system as illustrated in Fig. 2. In this ternary mobile phase gradients, all the peaks of the phytoalexins and the related indole metabolites were well resolved except those of brassitin (**7**), brassicanal A (**10**), brassilexin (**13**) and 1-methoxy-3-indolecarboxaldehyde (**19**) (Fig. 3E, standard sample). When the UV detector was operated at wavelengths $\geqslant 300$ nm, only brassicanal A (**10**) and 1-methoxy-3-indolecarboxaldehyde (**19**) could be detected since brassilexin (**13**) and brassitin (**7**) show weak UV absorption in this range. On the other hand, brassicanal A (**10**) and 1-methoxy-3-indolecarboxaldehyde (**19**) exhibit strong UV absorption in this range. For instance, brassicanal A (**10**) exhibits its absorption maximum at 311 nm (ε = 9740) [7], and 1-methoxy-3-indolecarboxaldehyde (**19**) at 300 nm (ε = 10 400) [24].

Using this method all cruciferous phytoalexins and related indole metabolites can be analysed in a single run within a reasonable time (50 min per run). Many samples can be analysed automatically, even at night, in combination with an automatic sampler. Reproducible chromatograms were obtained by maintaining the temperature of both the column oven and the solvent reservoirs at 35°C. Fig. 3 illustrates the HPLC chromatograms of the crude extracts obtained from control and *P. cichorii*-inoculated turnip tissues and Table I shows their phytoalexin contents. Accumulation of brassinin (**1**), methoxybrassinin (**2**) and cyclobrassinin (**4**) was clearly observed after 12 h of inoculation. Spirobrassinin (**6**) became the major phytoalexin at day 4, with a decline in brassinin (**1**) and cyclobrassinin (**4**). This result agrees with our biosynthetic studies: spirobrassinin (**6**) is biosynthesized from

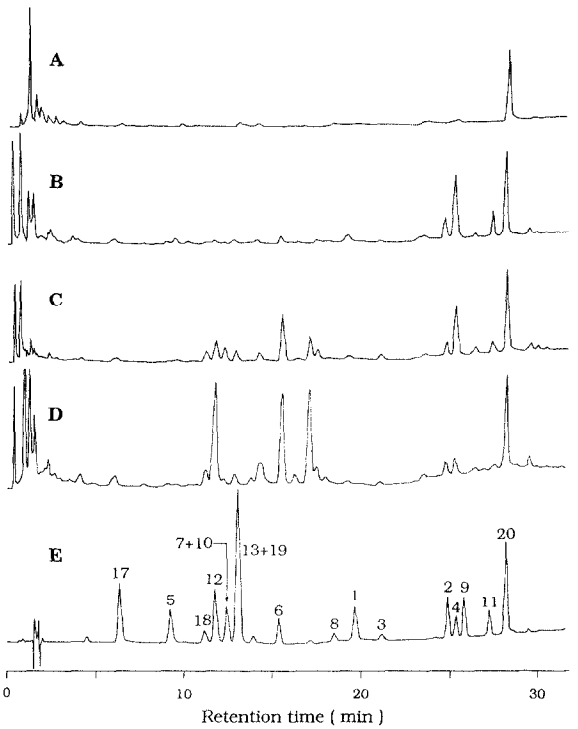

Fig. 3. HPLC of the crude extracts of turnip tissues and standard sample. Column, μBondapak C_{18}, 10 μm 300 × 3.9 mm I.D.; solvent system, acetonitrile–methanol–water; flow-rate, 2 ml/min; UV detection at 254 nm. (A) Control tissue after 3 days. (B) *P. cichorii*-inoculated tissue after 12 h. (C) Inoculated tissue after 2 days. (D) Inoculated tissue after 4 days. (E) Standard sample. Peaks: **1–13**, see Fig. 1; **17** = 3-indolecarboxaldehyde; **18** = 3-indolylacetonitrile; **19** = 1-methoxy-3-indolecarboxaldehyde.

TABLE I

PHYTOALEXIN CONTENTS OF *P. CICHORII*-INOCULATED TURNIP ROOT TISSUE

N.D. = Not detected. Each value represents the mean ± S.D. Experiments were repeated three times with three samples per experiment.

Time	Phytoalexin production (μg per g dried weight)			
	Spriobrassinin	Brassinin	Methoxybrassinin	Cyclobrassinin
Control (3 days)	N.D.	N.D.	N.D.	N.D.
12 h	10.1 ± 1.7	8.8 ± 1.0	20.0 ± 0.9	112.6 ± 8.9
2 days	61.9 ± 5.0	3.7 ± 0.4	10.9 ± 0.2	82.2 ± 3.5
8 days	130.2 ± 3.9	3.9 ± 0.4	14.0 ± 0.8	30.4 ± 2.0

brassinin (**1**) [25]. A detailed discussion of the time course studies of the phytoalexins is provided elsewhere [11].

Prior to this report, two groups have described procedures for the determination of cruciferous phytoalexins. Dahiya and Rimmer [26] have reported the HPLC analysis of methoxybrassinin (**2**) and cyclobrassinin (**4**) from elicited *B. napus* and *B. juncea* tissues and Kollmann *et al.* [27] have also demonstrated efficient clean-up of coloured plant extract with reversed-phase cartridges and reported the HPLC analysis of three phytoalexins. Recently, Rouxel *et al.* [28] reported the HPLC analysis of five indole phytoalexins [brassinin (**1**), methoxybrassinin (**2**), brassilexin (**13**), cyclobrassinin (**4**) and cyclobrassinin sulphoxide] using Kollmann's binary gradient system, and discussed the relationship between these indole phytoalexins and resistance to *L. maculans* within Brassicaceae. However, as they indicated in their paper, it seems difficult to be to determine polar phytoalexins such as brassilexin (**13**) using a simple gradient, since the slope of the methanol gradient is too steep. Furthermore, they did not analyse spirobrassinin (**6**), which is a major phytoalexin in Japanese radish (*R. sativus*) and turnip root (*B. campestris*), as illustrated in Fig. 3.

Recently, we have confirmed that spirobrassinin (**6**) and cyclobrassinin (**4**) are biosynthesized from brassinin (**1**) by incorporation studies of labelled compounds [25]. Devys and Barbier [29] suggested that brassilexin (**13**) is also biosynthesized from brassinin (**1**) via cyclobrassinin (**4**) and cyclobrassinin sulphoxide. In order to determine these biosynthetic pathways, spirobrassinin (**6**) and other polar and less polar phytoalexins must be analysed together with non-polar ones. Using our gradient system, thirteen phytoalexins from polar to non-polar and three related metabolites can be analysed in a single run.

Because Japanese radish and turnip roots were used as material, the analyses were not complicated by pigments, waxes, sterols and other interfering compounds, as illustrated in Fig. 3A (control tissue after 3 days). However, other coloured cruciferous plant materials could be also analysed by our method in combination with Kollmann's protocol. The combined method may be a standard analytical procedure of cruciferous phytoalexins.

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REFERENCES

1 J. A. Bailey and J. W. Mansfield, *Phytoalexins*, Blackie, Glasgow, 1982.
2 C. J. W. Brooks and D. G. Watson, *Natl. Prod. Rep.*, 2 (1985) 427.
3 H. D. Van Etten, D. E. Mathews and P. S. Mathews, *Annu. Rev. Phytopathol.*, 27 (1989) 143.
4 M. Takasugi, K. Monde, N. Katsui and A. Shirata, *Chem. Lett.*, (1987) 1631.
5 M. Takasugi, N. Katsui and A. Shirata, *J. Chem. Soc., Chem. Commun.*, (1986) 1077.
6 M. Takasugi, K. Monde, N. Katsui and A. Shirata, *Bull. Chem. Soc. Jpn.*, 61 (1988) 285.
7 K. Monde, N. Katsui, A. Shirata and M. Takasugi, *Chem. Lett.*, (1990) 209.

8 K. Monde, K. Sasaki, A. Shirata and M. Takasugi, *Phytochemistry*, 29 (1990) 1499.
9 K. Monde, K. Sasaki, A. Shirata and M. Takasugi, *Phytochemistry*, 30 (1991) 2915.
10 K. Monde, K. Sasaki, A. Shirata and M. Takasugi, *Phytochemistry*, 30 (1991) 3921.
11 K. Monde, M. Takasugi, J. A. Lewis and G. R. Fenwick, *Z. Naturforsch., C* 46 (1991) 189.
12 M. Devys, M. Barbier, I. Loiselet, T. Rouxel, A. Sarniguet, A. Kollmann and J. F. Bousquet, *Tetrahedron Lett.*, 29 (1988) 6447.
13 M. Devys, M. Barbier, A. Kollmann, T. Rouxel and J. F. Bousquet, *Phytochemistry*, 29 (1990) 1087.
14 J. S. Dahiya and S. R. Rimmer, *Phytochemistry*, 27 (1988) 3105.
15 T. Rouxel, A. Sarniguet, A. Kollmann and J. R. Bousquet, *Physiol. Mol. Plant. Pathol.*, 34 (1989) 507.
16 M. S. C. Pedras and J. L. Taylor, *J. Org. Chem.*, 56 (1991) 2619.
17 G. R. Fenwick, R. K. Heaney and W. J. Mullin, *CRC Crit. Rev. Food Sci. Nutr.*, 18 (1983) 123.
18 G. R. Fenwick, R. K. Heaney and R. Mawson, in P. R. Cheeke (Editor), *Toxicants of Plant Origin, Vol. II, Glycosides*, CRC Publishing, Boca Raton, FL, 1989, pp. 3–41.
19 R. McDannell, A. E. M. MacLean, A. B. Hanley, R. K. Heaney and G. R. Fenwick, *Food Chem. Toxicol.*, 26 (1988) 59.
20 P. Jandera and B. Prokes, *J. Liq. Chromatogr.*, 14 (1991) 3125.
21 T. Kowalska, *Chromatographia*, 29 (1990) 389.
22 A. Bartha, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 458 (1988) 371.
23 N. Tanimoto and M. Takasugi, unpublished results.
24 R. M. Acheson, P. G. Hunt, D. M. Littlewood, B. A. Murrer and H. E. Rosenberg, *J. Chem. Soc., Perkin Trans. 1*, (1978) 1117.
25 K. Monde and M. Takasugi, *J. Chem. Soc., Chem. Commun.*, (1991) 1582.
26 J. S. Dahiya and S. R. Rimmer, *J. Chromatogr.*, 448 (1988) 448.
27 A. Kollmann, T. Rouxel and J. F. Bousqet, *J. Chromatogr.*, 473 (1989) 293.
28 T. Rouxel, A. Kollmann, L. Boulidard and R. Mithen, *Planta*, 184 (1991) 271.
29 M. Devys and M. Barbier, *J. Chem. Soc., Perkin Trans. 1*, (1990) 2856.