Phytochemistry, Vol. 21, No. 4, pp. 917–922, 1982.
Printed in Great Britain.

0031-9422/82/040917-06$03.00/0

4-HYDROXYBENZOYLCHOLINE: A NATURAL PRODUCT PRESENT IN *SINAPIS ALBA*

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(*Received* 30 *July* 1981)

Key Word Index—*Sinapis alba*; Cruciferae; choline esters; sinapine; 4-hydroxy-3,5-dimethoxycinnamoylcholine; 4-hydroxybenzoylcholine; 3-hydroxybenzoylcholine; 2-hydroxybenzoylcholine; 4-hydroxy-3-methoxybenzoylcholine; 3-hydroxy-4-methoxybenzoylcholine; phenols; choline ester synthesis.

Abstract—The total fractions of choline esters have been isolated from different parts of *Sinapis alba*. 4-Hydroxybenzoylcholine has been identified and found to be one of the quantitatively dominating choline esters in seed extracts of *S. alba*. The identity of the new natural product has been confirmed by comparison with synthetic reference compounds. Several different choline esters are present in this plant but in extracts of seedlings, leaves, and inflorescences they are not so quantitatively dominating as in seeds. The modified ion-exchange technique applied appeared to be an efficient tool in the isolation and separation of choline esters and amines from other types of phenolic plant constituents.

INTRODUCTION

The choline ester of sinapic acid(4-hydroxy-3, 5-dimethoxycinnamoylcholine; sinapine) (**1**) is a well-known constituent of crucifer seeds ([1] and refs. cited therein). Feruoylcholine has been isolated from seeds of *Cleome pungens* (see below) and the isomeric 3-hydroxy-4-methoxycinnamoylcholine (**2**) as well as 2,4-dimethoxybenzoylcholine (hesperalin; **3**) are known plant products [2]. The major part of **1** in seeds of different crucifers has been shown to be rapidly transformed into other sinapic acid derivatives during the early stage of germination ([3] and refs. cited therein), but otherwise only little is known about the metabolism of choline esters in plants.

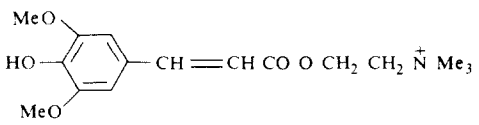
A relatively high concentration of **1** is known to occur in oilseed crops of cultivars of *Crambe* [4], *Sinapis* and *Brassica* [5, 6] as well as in seeds of the new double low rape varieties [7, 8]. Furthermore, relatively high concentrations of other bound low MW phenolic acids, e.g. 4-hydroxybenzoic acid, are known to be present in seeds of these plants as shown by the investigation of extracts subjected to base catalysed hydrolysis [5, 6]. Large amounts of **1** and possibly other choline esters of phenolic acids are undesirable in rapeseed meal used as a source of protein for food and feeding stuff [6, 9–11], and efforts have been directed towards the development of sensitive methods of analysis for screening rapeseed varieties for their choline ester content [7, 8].

The present work is a continuation of our previous studies of low MW constituents of crucifers [12–15], including rapeseed [16]. During these studies it was revealed that several fluorescent and UV-absorbing cationic compounds were present in extracts of the seeds. The present paper describes the isolation of the total fraction of choline esters present in different parts of *S. alba* by use of a modified ion-exchange technique. Among the quantitatively dominating choline esters in these fractions were **1** and the new natural product 4-hydroxybenzoylcholine (**4**).

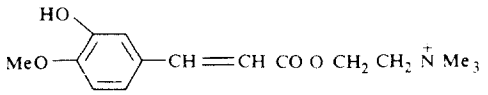
RESULTS AND DISCUSSION

The plant material was homogenized in boiling methanol–water to arrest enzyme catalysed degradations [17] and to increase the extraction efficiency. Investigations of the crude extract obtained from *S. alba* seeds were performed by PC and high voltage electrophoresis (HVE). It was revealed that *p*-hydroxybenzylamine (**4a**) [18], **1** and **4** co-occurred with several unknown compounds which have properties as choline esters according to PC, UV, HVE and reactions with Dragendorff's reagent [19].

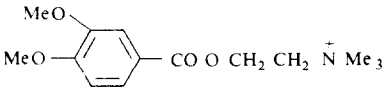
Attempts to use the strongly acidic cation exchange columns previously applied in the method of amine isolation [17] lead to serious problems with respect to complete elution of the intact choline esters from the columns. Therefore, the ion-exchange principles utilized in isolation of the strongly acidic intact glucosinolates from anion exchange columns [16, 17, 20] were adapted to the isolation of the quaternary ammonium ions using a weakly acidic cation exchanger. The concentrated extracts were applied to a column (Sephadex CM-25) with carboxymethyl groups in the protonated form. Neutral and acidic amino acids do not have a net positive charge at the pH used and so were not retained on the column whereas quaternary ammonium ions, e.g. **1** and **4**, basic amino acids, amines, e.g. **4a**, and the widely distributed plant constituent ethanolamine were retained. After thoroughly flushing the column with water, the elution was performed with 1 M acetic acid, which results in protonation of the carboxymethyl groups and removal of the charges on the column material. Thereby basic amino acids as well as other cations were released from the column in a small volume of



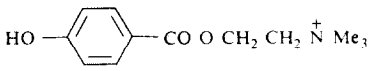
1 Sinapine



2 Isoferuoylcholine



3 Hesperalin



4

eluent, although phenolic choline esters, e.g. **1**, have a delayed elution owing to their relatively strong adsorption to the column material (see below). Evaporation of residues from the acetic acid eluates left the acetates of the ammonium ions which were further purified and separated into their individual compounds by preparative HVE and preparative PC.

The UV-data, chromatographic and HVE properties of isolated **1**, **4**, and **4a** are presented in Table 1 together with corresponding data for some reference compounds. Some of the data for **4a** and isomers thereof have been previously reported [17], and are included in Table 1 for comparison. The UV absorption spectrum [21] as well as other data previously reported for sinapine [2, 4] have been used to confirm the structure of **1**. The structure of the new natural product **4** has been confirmed by comparison of chromatographic, HVE, and spectroscopic data of the natural product (Tables 1 and 2) with corresponding data obtained with synthetic **4** prepared by well established methods [22] (see Experimental). Other choline esters have been prepared by the use of the same methods of synthesis. They have been used for chromatographic and spectroscopic comparison, but 3-hydroxybenzoylcholine (**5**), 2-hydroxybenzoylcholine (salicylcholine, **6**), 4-hydroxy-3-methoxybenzoylcholine (vanillylcholine, **7**), and 3-hydroxy-4-methoxybenzoylcholine (isovanillylcholine, **8**) are not known to be natural products.

The presence of **1** and **2** on chromatograms and pherograms is easily revealed by their strong but different fluorescence in long-wave UV light [2]. Correspondingly, the benzoic acid derivatives **3**–**8** produce less intense but different coloured spots in short-wave UV light. Characteristic but different colour changes on exposure to ammonia vapour are observed for the phenolic choline esters, colours which are slightly different from those of the corresponding carboxylic acids. This is in accordance with the differences found between the UV-spectra of the carboxylic acids and the corresponding choline esters (Table 1). With Dragendorff's reagent intense coloured spots are produced on the chromatograms and pherograms for all of the investigated choline derivatives [19].

Chemical shifts from ^{13}C NMR spectra of choline esters and some reference compounds are shown in Table 2. The previously reported ^{1}H NMR data for **2** [23] and feruoylcholine [24], the ^{13}C NMR data for some cinnamic acid derivatives [25], and the reported NMR data for different phenolic plant constituents [17] constitute, together with the values presented in Table 2, an efficient structural proof for the new natural product **4**.

Choline esters are easily hydrolysed in alkaline solutions and the ester bond in **6** is especially easily hydrolysed even at room temperature and in neutral aqueous solutions. The concentrations of choline esters as well as the carboxylic acids produced therefrom at different times of hydrolysis have been determined by UV-spectroscopy after separation of the compounds by HVE at pH 6.5. The released choline has been qualitatively detected on the pherograms by use of Dragendorff's reagent. GC and/or GC/MS determination of the carboxylic acids released by hydrolysis are efficient tools for identification purposes [6]. However, owing to instability of some phenols in alkaline conditions it is not a recommended method for quantitative analysis of all of the possible individual choline esters present in plant materials.

Table 3 shows the results obtained by determination of the contents of **1**, **4**, and **4a** in extracts from different parts of *S. alba* which have been purified by the described cation exchange technique (see above) and separated by preparative HVE before UV-determination (Table 1). The main parts of both **4** and **4a** are generally found in the acetic acid eluate (fractions 10–30) immediately after the basic amino acids and before the main part of **1** (fractions 20–70). The results have also revealed that some other quantitatively important cations present in the seed extract are other types of aromatic choline esters. This claim is based on comparison of the UV-spectra obtained after preparative HVE and the observation of different spots on the chromatograms and pherograms after treatment with Dragendorff's reagent. The results presented in Table 3 reveal, furthermore, that only relatively low concentrations of aromatic choline esters are present in green parts of *S. alba*, but that these plant parts contain quantitatively important amounts of as yet unidentified nonaromatic cations

Table 1. UV data, R_f-values and ionic mobilities of choline esters and some reference compounds

Compound	R_f values from PC in solvent*			Si gel R_f values from TLC in solvent*		Cellulose R_f values from TLC in solvent*		HVE mobilities in buffer system* (cm)		λ_{max} (nm) and corresponding (relative) E values‡ from UV spectra			
										In MeOH		In MeOH + MeONa	
	(1)	(2)	(3)	(1)	(4)	(1)	4	pH 1.9	pH 6.5				
4-Hydroxybenzoylcholine (4)	0.64	0.95	0.20	0.40	0.23	0.94	0.89	−15.7	−14.8	211(0.62)	262(0.37)	224(0.46)	302(0.58)
3-Hydroxybenzoylcholine (5)	0.58	0.95	0.24	0.39	0.30	0.91	0.89	−15.9	−15.4		305(0.50)		335(0.52)
2-Hydroxybenzoylcholine (6)	0.62	0.95	0.54	0.39	0.20	0.95	0.89	−16.7	16.0	241(1.2)	308(0.49)	247(0.78)	339(0.60)
Vanillylcholine (7)	0.60	0.95	0.17	0.32	0.22	0.90	0.87	−14.0	−13.6	232(1.0)	266(0.55)	232(> 1.5)	320(0.97)
											297(0.38)		
Isovanillylcholine (8)	0.57	0.95	0.15	0.29	0.19	0.86	0.85	−13.8	−14.5	218(1.2)	269(0.61)	234(1.5)	276(0.35)
											300(0.35)		333(0.30)
Hesperaline (3)	0.64	0.95	0.50	0.28	0.15	0.94	0.89	−13.9	−14.5	230(> 1.5)	265(0.68)	230(> 1.5)	264(0.68)
											295(0.46)		295(0.46)
3-Hydroxy-4-methoxycinnamoylcholine† (2)	0.62	0.95	0.18	0.34	0.16	0.94	0.72	−11.3	−11.7	230(> 1.5)	248(0.27)	230(> 1.5)	269(0.41)
										305(0.31)	333(0.35)	311(0.32)	374(0.19)
Sinapine† (1)	0.57	0.95	0.14	0.32	0.15	0.90	0.66	−10.1	−10.6	230(1.5)	245(0.4)	230(> 1.5)	260(0.70)
											335(0.43)		395(0.71)
Bromocholine§	0.48	0.95	0.45	0.18	0.27	0.79	0.98	−24.1	−25.5				
Choline	0.34	0.95	0.40	0.13	0.38	0.62	0.98	−25.9	−27.5				
Ethanoleamine	0.34	0.95	0.53	0.27	0.78	0.57	0.95	−28.5	−29.3				
4-Hydroxybenzylamine (4a)	0.56	0.93	0.56	0.60	—	0.85	—	−19.0	−18.6	278(0.87)	282(0.79)	242(> 1.5)	294(0.91)
Lysine	0.14	0.85	0.14	0.10	0.70	0.28	0.92	−21.8	−17.5				
4-Hydroxybenzoic acid	0.88	0.83	0.24	0.84	0.95	0.98	0.68	−0.5	10.6		256(0.9)		283(1.1)
3-Hydroxybenzoic acid	0.89	0.83	0.25	0.84	0.95	0.89	0.74	−0.5	11.0	235(> 1.5)	300(0.85)	240(> 1.5)	315(0.82)
2-Hydroxybenzoic acid	0.90	0.53	0.67	0.82	0.79	0.98	0.81	−0.5	12.5	231(1.05)	300(0.63)	245(> 1.5)	315(0.93)
Vanillic acid	0.81	0.66	0.18	0.89	0.85	0.98	0.63	−0.5	9.1	225(0.98)	260(0.86)	227(1.1)	285(1.07)
											295(0.42)		302(1.13)
Isovanillic acid	0.85	0.66	0.21	0.86	0.85	0.98	0.55	−0.5	8.9	225(1.05)	258(0.77)	232(1.5)	260(0.59)
											296(0.39)		317(0.32)
Sinapic acid†	0.80	0.85	0.19	0.85	0.95	0.98	0.30	−0.5	4.6	240(0.68)	322(0.68)	254(0.55)	365(0.80)

*For solvent and buffer systems, see Experimental.

†The *E*- (*trans*) isomer.

‡The absorbance values (relative *E*) show the relative intensities of the different peaks in the spectra. They do not show differences in absorbance between the compounds as different concentrations have been used to obtain appropriate spectra of the individual compounds. Quantitative determination of 1,4, and 4a have been based on the following UV values: (1) log $\epsilon = 4.30$ for the peak at 335 nm, (4) log $\epsilon = 4.20$ for the peak at 262 nm, and (4a) log $\epsilon = 3.20$ for the peak at 282 nm.

§(2-Bromoethyl)-trimethylammonium bromide.

Table 2. ^{13}C chemical shifts (δ) for different atoms of phenolic choline esters

	Choline moiety*			Carboxylic acid moiety*							
Compound	1	2	Me	1′	1″	2″	3″	4″	5″	6″	MeO—
4-Hydroxybenzoylcholine (**4**)	65.7	59.4	54.7	167.8	121.0	133.0	116.4	162.2	116.4	133.0	
3-Hydroxybenzoylcholine (**5**)	65.5	59.7	54.7	167.5	130.6	116.7	156.9	122.0†	131.0	1225†	
2-Hydroxybenzoylcholine (**6**)	65.4	59.7	54.6	169.7	120.1	160.6	118.1	137.4	121.1	131.0	
4-Hydroxy-3-methoxybenzoyl-choline (**7**, vanillylcholine)	65.6	59.5	54.6	167.8	121.2	113.7	148.0	151.6	115.9	125.2	56.6
3-Hydroxy-4-methoxybenzoyl-choline (**8**, isovanillylcholine)	65.5	59.5	54.6	167.6	121.7	116.6	145.6	153.3	112.2	124.3	56.6
Choline	68.3	56.4	54.7								

*2 Corresponds to the carbon atom connected to the ester oxygen atom, 1′ is the carbonyl carbon atom, Me is the methyl carbon atom connected to the ammonium group, MeO—is the methoxy group and those atoms marked″ are the aromatic carbon atoms.

†Values assigned these atoms may be interchanged.

Table 3. Contents of choline esters and 4-hydroxybenzylamine in different parts of *S. alba*

	Concentration (μmol/g plant material)*			
Compound	Seeds	Seedlings	Stems + leaves	Inflorescences
4-Hydroxy-3, 5-dimethoxycinnamoylcholine (sinapine; **1**)	33	3	†	†
4-Hydroxybenzoylcholine (**4**)	27	10	†	†
4-Hydroxybenzylamine (**4a**)	5	7	3	8

*Determined by UV spectroscopy after isolation as described in Experimental.

†The amount isolated was too small in relation to other compounds present in the isolated fraction to allow a reliable quantitative determination.

(see Experimental). This is in accordance with the reported observation for various Cruciferae members of a rapid transformation of the seed constituent **1** into other products during the early stage of germination [3].

The group of choline esters known as plant products now comprise the three cinnamic acid derivatives **1** [1], **2** [2] and feruoylcholine [24] and the two benzoic acid derivatives **3** [26] and the new natural product **4**. Few reports are available on the biosynthesis of these esters in plants [27, 28]. With the results now presented it seems probable that several other choline esters will be found in crucifer seeds. It is well-known that the relatively high concentration of different bond phenolic acids occurring in Cruciferae seeds [4–6] are actively metabolized during germination [3] (see above). The concentration ratio observed between 4-hydroxybenzoic acid and sinapic acid in alkaline hydrolysates of extracts from *Brassica* and *Sinapis* oilseeds [4, 5] is in accordance with the concentration ratio now reported for **1** and **4** in corresponding extracts of *S. alba* seeds. Furthermore, the reported co-occurrence of these compounds with appreciable amounts of other bound phenolic acids [4, 5] is of special interest in connection with the observation of other as yet unidentified choline esters in extracts of *S. alba* seeds (see above).

EXPERIMENTAL

Plant material. Seeds of *Sinapis alba* L. (white mustard) were obtained from Trifolium Silo A/S, DK-2630 Tåstrup, Denmark. The plants were grown in the greenhouse, and immediately after harvesting the plant material was lyophilized and stored at −20°.

General methods and instrumentation. Methods and equipment used for GC, MS, ^{1}H NMR, ^{13}C NMR, HVE and PC have been described elsewhere [16]. PC was performed on Whatman No. 1 paper in solvents: (1) *n*-BuOH–HOAc–H_2O (12:3:5); (2) PhOH–H_2O–13 M NH_4OH (120:30:1) (w/v/v) and (3) *iso*-PrOH–H_2O–13 M NH_4OH (8:1:1). HVE was carried out on Whatman 3 MM paper in buffer systems: (1) pH 1.9 (HOAc–HCO_2H–H_2O (4:1:45), 1 hr at 3.2 kV and 90 mA and (2) pH 6.5 (pyridine–HOAc–H_2O) (25:1:500), 30 min at 5 kV and 90 mA. TLC was performed on Si gel plates (DC-Alufolien; Kieselgel 60 F_{254}; 20 × 20 cm; Merck) and on cellulose plates (DC-Alufolien; Cellulose F_{254}; 20 × 20 cm; Merck) in solvents: (1) and (4) HOAc–H_2O (6:94).

Isolation of choline esters. The previously described methods [17] were used for homogenization and extraction. The MeOH–H_2O extract (3 × 50 ml) from seeds of *S. alba* (10 g) was taken to dryness, extracted with $CHCl_3$ (3 × 10 ml), leaving an evaporation residue (1.763 g). The residue was dissolved in H_2O (50 ml) and applied to a column (1.5 × 30 cm) of Sephadex CM-25 in the H^+-form. The column was flushed with H_2O (3 l.) after which the retained cations were eluted from the column with 1M HOAc. Fractions (15 ml) were collected at 40 ml/hr. Fractions; 1–8 (87 mg), 9–20 (225 mg), 21–40 (93 mg), 41–105 (40 mg), 106–155 (4 mg) contained the basic amino acids (lys, arg, his), amines (**4a**), and choline esters (**1** and **4**) as mentioned in the Results and Discussion.

Correspondingly, lyophilized seedlings (10 g; 3 × 200 ml MeOH–H_2O) (treated as for the seeds) produced an evaporation residue (3.998 g) and the following fractions; 4–10 (120 mg), 11–19 (346 mg), 20–29 (137 mg), 30–60 (233 mg). Lys, arg, his, amines (**4a** and ethanolamine), and choline esters were eluted as mentioned above for the seeds. The leaves (10 g) (treated as for the seeds) produced an evaporation residue (3.970 g) and fractions; 3–60 (942 mg) containing basic amino acids and the amines mentioned for the seedlings but only trace amounts of choline esters. From inflorescences (8.8 g) (treated as for the seeds) an evaporation residue (3.00 g) and the following fractions were obtained; 3–16 (640 mg), 17–60 (222 mg) containing basic amino acids and amines as for the leaves and correspondingly without reliable detection of choline esters (see Table 3).

The choline esters were separated by prep. HVE in buffer (2) pH 6.5 and prep. PC in solvents (1) and (3). Compounds **1**, **4**, and **4a** were identified by well established methods and the results from PC, TLC, HVE, UV, and ^{13}C NMR are shown in Tables 1 and 2. The ^{1}H NMR spectra of the acetates exhibited signals at the following values: **1**, 7.7 (1H, *d*), 7.0 (2H, *s*), 6.4 (1H, *d*) 4.5 (2H, *m*), 3.7 (2H, *m*), 3.2–3.3 (15H), 1.95 (3H, *s*); **4**, 7.0–7.4 (4H), 4.5 (2H, *m*), 3.7 (2H, *m*), 3.2 (9H), 1.95 (3H).

Hydrolysis of the total choline ester extract from the seeds of *S. alba* and of the preparatively isolated **1** and **4**, respectively, was performed in 2 M NaOH (5 ml) on a boiling water bath. The evaporation residues of the hydrolysis mixtures were dissolved in 4 M HCl (5 ml), taken to dryness and treated with EtOAc (3 × 5 ml). The carboxylic acids obtained by evaporation of the EtOAc extracts were identified by PC, TLC, HVE, GC and UV.

Synthesis of silver carboxylates. The carboxylic acids (10 mmol) were dissolved in H_2O (10 ml) by adding NaOH (2 M) to pH 10. The solns were taken to pH 6.5 with HNO_3 (2 M) and by addition of $AgNO_3$ (10.1 mmol), crystals of the silver carboxylates were obtained. The crystals were filtered off, washed with H_2O (2 × 3 ml) and EtOH (2 × 5 ml), and dried, resulting in the following yields: 2.46 g (100%) silver 4-hydroxybenzoate, 1.95 (80%) silver 3-hydroxybenzoate, 2.32 g (95%) silver 2-hydroxybenzoate, 2.00 g (75%) silver 4-hydroxy-3-methoxybenzoate, 2.54 g (93%) silver 3-hydroxy-4-methoxybenzoate.

Synthesis of choline esters. Bromocholinebromide (1 mmol, 247 mg) and the crude silver carboxylates (1 mmol of each, respectively), dissolved in H_2O (10 ml) were treated as described elsewhere [22]. AgBr was removed by filtration and the filtrates were taken to dryness after which the evaporation residues were heated to 100° for 7–8 hr [22]. The reaction products thus obtained were dissolved in 1:1 mixtures of MeOH–DMSO (5 ml) and applied to Sephadex CM-25 columns (1.2 × 6 cm) in the protonated form. The columns were washed with H_2O (300 ml) and eluted with 2 M HOAc. Evaporation of the HOAc eluates (125 ml) left greasy semicrystalline acetates of the choline esters in the following yields: 206 mg **4** (73%), 220 mg **5** (78%), 270 mg **6** (95%), 236 mg **7** (75%) and 213 mg **8** (68%). The bromocholine and choline in these fractions were easily separated from the choline esters by prep. HVE (Table 1). Identity of the compounds was established by PC, TLC, HVE, UV, ^{1}H and ^{13}C NMR (Tables 1 and 2).

Acknowledgements—We wish to thank Professor R. Gmelin for reference samples of compounds **2** and **3** and Dr. K. Schaumburg, University of Copenhagen, for help with the NMR spectra. Support from the Danish Agricultural and Veterinary Research Council is gratefully acknowledged.

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