Synthesis and Characterisation of Acyl Coenzyme A Derivatives of Aromatic Carboxylic Acids

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Coenzyme A Thioesters, Aromatic Carboxylic Acids

Acyl CoA derivatives of a number of cinnamic and benzoic acids have been synthesised *via* the appropriate acyl phenyl thiol esters. The reaction has potential application in the preparation of acyl CoA derivatives of radiolabelled aromatic carboxylic acids. UV absorption spectra have been determined for the CoA thiol esters following their purification by gel permeation chromatography. By comparison of the extinction in the UV region with that produced at 540 nm during the hydroxamate assay of Lipman and Tuttle, it has been possible to determine the UV molar extinction coefficients for the series.

Coenzyme A (CoA) esters of cinnamic acid, benzoic acid and their hydroxylated and methoxylated derivatives have recently attracted attention [1, 2] by their demonstrated involvement in the biosynthesis of secondary plant metabolites, notably flavonoids and lignin. Hahlbrock [3] has used the mixed anhydride method of Stadtman [4] for the preparation of cinnamoyl CoA itself, and this procedure has also been applied in the synthesis of other non-hydroxylated cinnamic acids [5]. Gross and Zenk [6] have prepared a number of CoA thiol esters of hydroxylated cinnamic acids, using an acyl CoA-synthetase from ox-liver, and Hahlbrock *et al.* [1] have achieved similar results with an enzyme from soya bean. However, both these enzymes have specificity limitations. We now describe an adaptation of Trams and Brady's malonyl CoA synthesis [7] to the general preparation of CoA thiol esters of phenolic carboxylic acids. Furthermore, the spectral data for the compounds synthesised have been re-evaluated and in some cases supercede those obtained by Gross and Zenk [6]. A scaled down version of the reaction is suitable for the production of thiol esters from radiolabelled acids. Comparative studies with methoxylated aromatic acids have provided further confirmation of the reliability of the method.

Materials and Methods

Solvents and chemicals were regular commercial grades. Dimethyl formamide used for all experiments was dried by distillation from calcium hydride at atmospheric pressure under nitrogen and stored over molecular sieve. Triethylamine was distilled from and stored over potassium hydroxide pellets. Chloroform was distilled from calcium chloride, and benzene was dried over sodium wire.

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Synthesis of phenyl thiol esters of phenolic acids [7]

The acid in question (1 mmol) and thiophenol (1.1 mmol) were dissolved together in dimehylformamide (5 ml). This solution was then stirred in an ice bath for one hour while a solution of dicyclohexyl carbodiimide (DCC) (600 mg) in dry dimethylformamide (5 ml) was added dropwise. Water (10 ml) was then added, and stirring continued for 15 min, after which the mixture was made slightly acid and extracted with ether (3×20 ml). The ether phase was washed with 0.01 M hydrochloric acid and with water, dried over sodium sulphate and evaporated to dryness. The gummy residue still contained much dicyclohexylurea which could not be removed, but thiol ester exchange was found to be practicable without further purification.

Synthesis of phenyl thiol esters on non-hydroxylated aromatic acids

The following is a more mild procedure than that of Wieland and Köppe [8]. An appropriate unsubstituted or alkoxylated aromatic carboxylic acid (2 mmol) was refluxed in dry benzene (5 ml) with thionyl chloride (1 ml) for half an hour. Solvent and excess reagent were then removed *in vacuo* and the product acyl chloride was dissolved in chloroform (5 ml). This solution was then stirred in an ice bath while a solution of thiophenol (2 mmol) and dry triethylamine (2.2 mmol) in chloroform (5 ml) was added dropwise over 1 hour.

Moisture was excluded throughout the operation. After a further hour at room temperature, the chloroform solution was washed twice with 5% sodium bicarbonate solution and twice with water (each $\times$ 10 ml), dried over sodium sulphate and the solvent removed *in vacuo*. The product generally crystallised completely when excess thiophenol had been removed by leaving overnight at 0.1 mm mercury and room temperature. Except in the case of benzyloxycinnamoyl derivatives, all phenyl thiol esters were free of the parent acids by this stage, and were essentially pure (thin layer chromatography (TLC) silica; benzene plus 10 or 20% ethyl acetate). Crude yields ranged from 85 – 95%. Recrystallisation was from methanol or benzene/hexane, and recovered pure yields were variable, lying in the range 60 – 80%. Methanol gave the best results but gave lower yields, apparently causing some methanolysis of the product. Prolonged contact with hot methanol (more than 10 min at reflux) or cold methanol (more than 12 hours) caused noticeable loss of product with formation of the methyl ester. Analytical data and melting points observed for the products are recorded in Table I.

Table I. Melting points and analytical data for non-hydroxylated series of phenyl thiol esters.

Phenyl thiol ester	m.p. [°C]	Analysis [%] Calcd	Found
Benzoyl	55 – 56 *	C 72.87	C 72.58
		H 4.70	H 4.60
		S 14.47	S 15.28
Cinnamoyl	90 – 91 **	C 74.47	C 75.50
		H 5.03	H 5.54
		S 13.34	S 13.24
4-Methoxycinnamoyl	92 – 93	C 71.09	C 70.75
		H 5.22	H 5.15
		S 11.86	S 10.77
3,4-Dimethoxy-cinnamoyl	116 – 117	C 67.98	C 68.19
		H 5.37	H 5.45
		S 10.67	S 10.77
3,4,5-Trimethoxy-cinnamoyl	80 – 81	C 65.45	C 65.21
		H 5.45	H 5.64
		S 9.68	S 10.01
4-Benzyloxycinnamoyl	97 – 99	–	–
3,4-Dibenzyloxy-cinnamoyl	121 – 123	–	–

* Literature value 55 – 56 °C [13].
** Literature value 92 – 93 °C [14].

Thiol ester exchange [7, 9]

CoA (10 μmol) was dissolved in 0.1 M sodium bicarbonate buffer (2 ml) and the solution was cooled in ice. A solution of the thiol ester (10 μmol or excess) in methanol (1 ml) was added and the solution was either:

a. Stirred in ice for 3 hours while a stream of nitrogen was bubbled through, or b. sealed under nitrogen and shaken in the cold room for 3 hours.

At the end of this time the solution was acidified with 98% formic acid (50 μl) and extracted three times with its volume of ether. Yields, estimated spectroscopically (UV), ranged from approx. 80% in the case of the non-hydroxylated acid derivatives (from prepurified phenyl thiol esters) to approx. 30% for most of the hydroxylated derivatives. A very low yield of *o*-coumaroyl CoA made determination of the ε_{max} for this compound impracticable.

Hydroxamate test [10]

Testing of acyl CoA solutions was accomplished by treating 50, 100, and 150 μl aliquots of the solution with 0.2 M neutralised hydroxylamine (pH 8.0, 50 μl), making the solution up to 200 μl with water as necessary, and allowing the mixture to react at 30 °C for 10 min. After treatment with 100 μl of a solution containing ferric chloride (2.5 g) and trichloroacetic acid (10 g) in 1 N hydrochloric acid (10 ml), followed by centrifugation, absorption was measured in microcuvettes in a colourimeter at 546 nm against a suitable blank. A scan of several wavelengths around this value gave the shape of the absorption peak.

Purified phenyl thiol esters were weighed directly (approx. 1 mg) into centrifuge tubes, dissolved in methanol (0.5 ml) and treated with 0.2 M hydroxylamine (pH 8.0) for 10 min at 30 °C. Water (1 ml) was then added, followed by the above-mentioned acid ferric chloride reagent (1 ml). The mixture was shaken vigorously, centrifuged and transferred to a 3 ml cuvette in which the region 700 – 400 nm was scanned against a blank.

Microscale preparation of CoA thiol esters of phenolic acids

The method was used for *p*-coumaric acid and for ferulic acid. a. A solution of the acid in question (10 μmol) was dissolved in dry dimethylformamide (50 μl), thiophenol (1 μl, approx. 10 μmol) was added and the mixture was cooled in ice. DCC (6.0 mg) in dimethylformamide (50 μl) was added at a rate of 5 μl solution every 5 min. After each addition the mixture was shaken well and then returned to the ice bath. After the last addition the mixture was treated with water (50 μl), shaken, and allowed to stand for 15 min. It was then partitioned between water (10 ml) and ether (3 $\times$ 10 ml). The ether layers were washed with dilute acid and with water as described above, dried over sodium sulphate, evaporated to dryness and used immediately for thiol ester exchange.

b. Thiol ester exchange was carried out as described above, using the theoretical stoichiometrical requirement of CoA (10 μmol). Yields were only approx. 15%, presumably reflecting poor thiol formation as a consequence of insufficient mixing during the preceding DCC reaction.

Purification of CoA derivatives

The method of Hahlbrock *et al.*[1] using column chromatography on Sephadex G 10 gel was employed. Dilute formic acid (0.05 M; pH ca. 2.5) was used as solvent. Total gel bed volume was 300 ml. General monitoring of peaks was carried out by a "Uvicord" fraction collector. The fraction of interest, containing both adenine and thiol ester UV absorption peaks occurred in every case in the 90 – 120 ml elution. A peak containing only adenine absorption occurred between 60 – 70 ml elution volume. A smaller peak with thiol ester absorption but no adenine peak came at about 200 ml elution volume and may be due to thiol ester exchange with pantetheine or some other artifact, possibly formed by partial hydrolysis of CoA in the bicarbonate buffer used for the exchange. Additional monitoring was obtained by scanning whole fractions at regular intervals in a Leitz-Unicam UV spectrometer. Yields of purified CoA thiol ester (by UV) were in the range 50 – 70% of the crude product.

Results and Discussion

The reaction of phenolic acids with thiol in the presence of DCC produces a suitable intermediate for thiol ester exchange with CoA in the presence of bicarbonate buffer. As would be expected the thiol group attacks the intermediate formed by the action of DCC more readily than the phenolic groups present in the acid side chain and thus a phenyl thiol ester is formed, rather than a polymer caused by head-to-tail esterification of the acids. Attempts to isolate phenolic phenyl thiol esters from the crude DCC reaction mixtures all proved unsuccessful, since exposure to most adsorption chromatography media for any length of time resulted in considerable degradation of these compounds. However, the presence of phenyl thiol esters of all the phenolic acids examined could be demonstrated by thin layer chromatography on silica gel. Benzene : ethyl acetate (9 : 1 or 8 : 2) were found to be the best solvent systems. Phenyl thiol esters gave a yellow or greenish fluorescence under UV light (350 nm). The parent acids all gave blue fluorescence of various shades, or none at all. R_F values for the phenyl thiol esters studied were as in Table II.

Table II. R_F values of phenyl thiol esters and their parent acids on silica gel. I: in benzene/ethyl acetate 9 : 1, II: in benzene/ethyl acetate 8 : 2.

Parent acid	R_F of parent acid		R_F of phenyl thiol ester	
	I	II	I	II
Benzoic	—	0.41	—	0.92
4-Hydroxybenzoic	0.05	—	0.28	—
Protocatechuic	0.02	—	0.16	—
Cinnamic	0.43	—	0.84	—
o-Coumaric	0.10	—	0.30	—
m-Coumaric	0.05	—	0.21	—
p-Coumaric	—	0.10	—	0.69
p-Methoxycinnamic	—	0.21	—	0.85
3,4-Dimethoxycinnamic	—	0.10	—	0.59
Ferulic	—	0.16	—	0.70
Caffeic	—	0.05	—	0.32
3,4,5-Trimethoxycinnamic	—	0.10	—	0.60
Sinapic	—	0.08	—	0.51
4-Benzyloxycinnamic	—	0.18	—	0.91
3,4-Dibenzyloxycinnamic	—	0.13	—	0.89

If the developed plate was exposed to the atmosphere for a few hours the phenyl thiol ester spots (but not the acids or dicyclohexylurea) appeared yellow in visible light.

A series of phenyl thiol esters was prepared from benzoic acid and cinnamic acid and its alkoxylated derivatives. Reaction of the acyl chlorides with thiophenol in the presence of triethylamine gave good yields of the thiol esters. All of the products were obtained crystalline, and their spectral data are shown in Table III. The phenyl thiol esters of cinnamic and benzoic acid have previously been reported[11, 12], but no mention has been found in the literature of any of the other derivatives prepared. A list of analytical data are shown in Table I for all isolated compounds used in the thiol ester exchange reaction.

Treatment of solutions of CoA in bicarbonate buffer with phenyl thiol esters prepared by either the DCC or the acyl chloride method gave the corresponding CoA thiol esters. These products could not be extracted from acidic solution with ether, and exhibited for the most part the same λ_{max} values as reported by Gross and Zenk[6].

Wieland and Rueff[9] have described the thiol exchange reaction shown in Scheme 1 as essentially irreversible when R′ = alkyl.

Scheme 1:

$$R-CO\cdot S-Ph + R'-SH \rightleftharpoons R-CO\cdot S-R' + Ph-SH$$

Table III. Spectroscopic data for the non-hydroxylated aromatic acids and their phenyl thiol esters.

Parent acid	Acid (UV)		Phenyl thiol ester		
	λ_{max} [nm]	$\varepsilon_{max} \times 10^3$ [cm²/mol]	λ_{max} [nm]	$\varepsilon_{max} \times 10^3$ [cm²/mol]	IR [cm⁻¹]
Benzoic	226 272	5.9 0.82	238 266	14.6 8.0	1670, 1440, 1210, 900, 753
Cinnamic	270	19.3	303	14.4	1680, 1330, 1040, 1020, 885, 753
4-Methoxycinnamic	290–304	18.8	329	31.5	1670, 1240, 1160, 1030, 805, 750
3,4-Dimethoxycinnamic	292 317	14.8 17.3	343	21.0	1650, 1480, 1120, 1020, 809, 754
3,4,5-Trimethoxycinnamic	291	14.3	320	22.6	1680, 1430, 1120, 1020, 833, 746
4-Benzyloxycinnamic	287	16.6	312	33.0	1690, 1500, 1160, 1020, 818, 752
3,4-Dibenzyloxycinnamic	295 319	14.8 16.2	337	20.3	1680, 1490, 1130, 1020, 800, 750

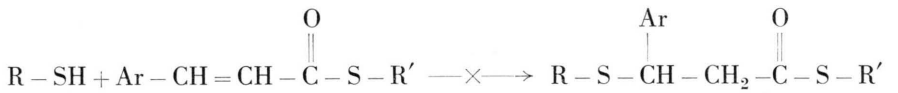
Thus yields of CoA thiol ester, which were all approx. 30% for products of the DCC reaction probably give a good indication of phenyl thiol ester yields from the latter process.

When thiophenol was added to solutions of several acyl CoA derivatives in bicarbonate buffer at 0 °C, no loss of UV absorption was observed within the critical range 280 – 360 nm. There is therefore no evidence that Michael addition of a thiol to the double bond of an acryloyl thiol ester, mentioned by Stadtman [4], occurs in the cinnamoyl homologues under the conditions used (Scheme 2).

Scheme 2:

a.

b.



c.

The transformation shown in Scheme 2c has been accomplished by Tanaka and Yokoyama [13] in poor yield (10%) by treating cinnamyol phenyl thiol ester with triethylamine in liquid hydrogen sulphide.

The acyl CoA derivatives were separated from excess CoA and other impurities by gel permeation chromatography on Sephadex G 10 in 0.05 M formic acid, as described by Lindl, Kreuzaler, and Hahlbrock [1]. Fractions were monitored by scanning the UV spectrum and only those which had a constant ratio of adenine/thiol ester absorption were retained.

It was desirable to further establish the identity of the acyl CoA derivatives obtained. Paper and cellulose thin layer chromatography in various solvent systems was tried but only the acetate-buffer/ethanol system gave detectably different R_F values for the various derivatives, in contrast to the findings of Gross and Zenk [6]. All did however give a weak delayed nitroprusside reaction [14]. The hydroxamate colour test [10] was used to assay the acyl CoA derivatives and to cross-check the ε_{max} values of Gross and Zenk. The hydroxamate test was first calibrated using the purified phenyl thiol esters. Thiophenol itself produced no change in the spec-

trum of acified ferric chloride solution. Gross and Zenk report an extinction coefficient of 1.54×10^6 cm^2/mol for cinnamoyl hydroxamic acid, which is presumably a misprint as a value of only 1.54×10^3 cm^2/mol is equivalent to their allowance of 0.513 extinction for 1 μmol in 3 ml solvent. Mean readings for our series of phenyl thiol esters lead to a value of 1.05×10^3 cm^2/mol, wich is more in line with that found for acetylhydroxamic acid (0.975×10^3). Variations of 10% are not uncommon in these determinations. All samples were scanned, and maxima varied slightly within the range 520 – 570 nm even for the same phenyl thiol ester. Closely similar absorption curves were found on testing the acyl CoA derivatives. Taking the extinction coefficient for the hydroxamate test to be 1.05×10^3 cm^2/mol, it was possible to estimate λ_{max} values for all the CoA thiol esters prepared. Complete UV data for the compounds are as shown in Table IV.

Gross and Zenk's extinction coefficient values were apparently obtained by allowing an acyl CoA-synthetase reaction to proceed to completion. My evidence shows that in some cases the reaction may not have gone to completion. An explanation of discrepancies in λ_{max} between the figures obtained this work and those of Gross and Zenk may involve the question of *cis*-, *trans*-isomerism of the acyl group. Conceivably the ligase enzyme could preferentially convert one isomer, whereas the chemical reaction should be less specific. No explanation is available as to why Gross and Zenk should have obtained higher ε values for cinnamoyl and 3,4-dimethoxycinnamoyl CoA than were observed in the above work. As was indicated in discussion of the hydroxamate test, accuracies are not likely to be greater than $\pm 10\%$. It will be noticed however, that the absorption maxima and values for the acyl CoA derivatives are closely similar to those for the equivalent purified phenyl thiol esters, and this is taken as an encouraging sign of the reliability of the CoA thiol ester results.

Yields of column-purified acyl CoA were estimated at 50 – 70%, based on CoA. Where a small-scale phenyl thiol ester preparation was carried out using DCC, final yields based on acid were about 10%. In the case of *o*-coumaric acid, very poor yields were obtained, and for this reason insufficient material was available for the extinction coefficient to be established.

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Table IV. UV Absorption data for aromatic acyl CoA derivatives (measured at pH 2.5 – 3.0).

CoA Derivative	λ_{max} I [nm]	λ_{max} II [nm]	ε_{max} II $\times 10^3$ [cm^2/mol]	$^*\lambda_{max}$ II [nm]	$^*\varepsilon_{max}$ II $\times 10^3$ [cm^2/mol]
Benzoyl	257	sh. 245	—	—	—
4-Hydroxybenzoyl	257	sh. 290	—	—	—
Protocatechuyl	254	—	—	—	—
Cinnamoyl	257	312	11	311	22
o-Coumaroyl	257	340	—	351	—
m-Coumaroyl	257	303	25	330	11
p-Coumaroyl	257	333	35	351	23
p-Methoxycinnamoyl	257	330	28	339	27
Caffeoyl	257	347	19	363	13
Feruloyl	257	347	26	345	19
iso-Feruloyl	257	346	29	351	18
3,4-Dimethoxycinnamoyl	257	345	20	346	24
Sinapoyl	257	352	28	—	—
3,4,5-Trimethoxycinnamoyl	257	335	27	342	9

* Figures obtained by Gross and Zenk [6].

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