Synthesis of Piperoyl Coenzyme A Thioester

Ursula Semler*, Günter Schmidtberg**, and Georg G. Gross*

* Universität Ulm, Abteilung Allgemeine Botanik, Oberer Eselsberg, D-7900 Ulm, Bundesrepublik Deutschland

** Universität Ulm, Sektion Massenspektrometrie, Oberer Eselsberg, D-7900 Ulm, Bundesrepublik Deutschland

Z. Naturforsch. **42c**, 1070–1074 (1987); received June 25, 1987

Piperic Acid, 5-(3,4-Methylenedioxyphenyl)-2,4-pentadienoic Acid, Piperoyl Coenzyme A, Thioester, N-Succinimidyl piperate, Piperoyl Hydroxamic Acid

Piperoyl-CoA, a potential intermediate in the biosynthesis of piperine, the pungent principle of pepper, was synthesized *via* N-succinimidyl piperate and subsequent transesterification with CoA-SH. UV-spectrophotometry of the HPLC-purified compound revealed, besides a maximum at 260 nm, a pronounced second absorption maximum at 368 nm due to the thioester linkage for which, as determined *via* the Fe^{3+}-complex of piperoyl hydroxamate and with [^{14}C]piperoyl-CoA, a molar extinction coefficient of 30.8×10^6 [$cm^2 mol^{-1}$] was calculated.

Introduction

It has long ago been suggested that the alkaloid piperine, the pungent principle of pepper *(Piper nigrum, P. longum)*, is synthesized in plants from piperidine and piperoyl-CoA as the activated acyl-moiety [1]. The correctness of this assumption still awaits proof, however, supporting evidence arose from the isolation of enzymes from barley [2, 3] and tobacco [4] that catalyzed an analogous formation of acid amides from cinnamoyl-CoA thioesters and various biogenic amines. With respect to piperine, it was thus decided to synthesize and characterize the still unknown CoA-derivative of piperic acid as a prerequisite for studies on the biosynthesis of this natural product.

Experimental

Analytical methods

The purity of synthesized compounds was checked by TLC on silica-gel plates in the following systems: (1) toluene–ethyl formate–HCOOH = 5:4:1; (2) $CHCl_3$–EtOAc–C_6H_6–MeCOEt–light petrol = 7:1:2:3:3; (3) $CHCl_3$–MeOH–*n*-hexane = 65:10:15; (4) C_6H_6–EtOAc–HOAc = 85:14:1; (5) *n*-hexane–Et_2O = 6:4; (6) 5% aq. HCOOH (cellulose MN 300). Hydroxamic acids were stained with acidic $FeCl_3$-solution [5], thioesters and free CoA-SH by the "delayed" nitroprusside reaction [6].

Analytical HPLC of piperoyl-CoA and related derivatives was done by an adaptation of the method given in ref. [7]; for details, see Fig. 2.

Mass spectra were recorded on a Varian MAT 711. Melting points (uncorrected) were determined microscopically with a Kofler micro heat bench. C,H,N-analyses were performed by Sektion Analytik und Höchstreinigung, Universität Ulm, and by Mikroanalytisches Laboratorium I. Beetz, Kronach.

Chemicals

Piperic acid was obtained by hydrolysis of piperine (Aldrich) [8] as pale yellow needles, m.p. 224 °C (lit. 216–217 °C), recovery 82%. The product was pure as judged in TLC system (1) and gave the expected signals upon EI-MS. 3,4-Methylenedioxycinnamaldehyde was prepared by reacting piperonal with acetaldehyde [9], yielding pale yellow crystals from EtOH-light petrol, m.p. 85 °C (lit. 84–85 °C); purity and identity of the aldehyde were confirmed by TLC (system 5) and EI-MS. Redistilled neutralized hydroxylamine was prepared according to ref. [10].

Chemical syntheses

[2-^{14}C]Piperic acid

3,4-Methylenedioxycinnamaldehyde (100 μmol, 17.6 mg) and 3.7 MBq (100 μCi) [2-^{14}C]malonic acid (NEN-DuPont; 100 μmol, 10.4 mg) were dissolved in 0.5 ml dry pyridine containing 10 μl piperidine

Abbreviations: CoA, CoA-SH, coenzyme A; DCC, N,N′-dicyclohexyl carbodiimide; DCU, N,N′-dicyclohexyl urea; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

Reprint requests to Prof. Dr. G. G. Gross.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0900–1070 $ 01.30/0

and heated for 4 h at 85 °C. After cooling, the sample was diluted with water, acidified with 5 N HCl and extracted 10-times with EtOAc. TLC (systems 1, 3, 4) of the organic phase revealed the formation of labeled piperic acid as minor component, together with large amounts of a second substance that was identified by TLC and EI-MS as 2-carboxypiperic acid (M^+ 262) [9]. The products were purified by semipreparative HPLC (Merck Lichrosorb Si-60, 7 μm, column 250×7 mm, solvent: toluene–EtOAc = 75:25, flow rate 2 ml/min). The dicarboxylic acid was subjected to thermolysis in quinoline at 160 °C for 20 min [9, 11] resulting in the almost quantitative conversion to piperic acid as shown by TLC (systems 1, 4) after working up the reaction mixture as described above. The combined two fractions of piperic acid gave a total recovery of 54%.

N-Succinimidyl piperate

To a stirred solution of piperic acid (5 mmol, 1.09 g) and N-hydroxysuccinimide (5 mmol, 0.58 g) in 30 ml dioxane was added solid DCC in small portions. After stirring overnight, precipitated DCU was removed by filtration of the resulting yellow suspension. The filtrate was evaporated *in vacuo* (40 °C); the dried residue was dissolved in boiling $CHCl_3$ and crystallized by addition of light petrol as yellow needles, m.p. 230–232 °C in 45% yield. The product was pure as shown by TLC (solvents 1, 2). Elementary analysis gave 60.94% C; 4.16% H; 4.42% N (calcd. for $C_{16}H_{13}NO_6$: 60.95% C; 4.16% H; 4.44% N). MS (70 eV): *m/z* 315 (18%, M^+), 201 (100, M − succinimidyl), 173 (4), 171 (11, 201 u − CH_2O), 143 (11, 171 u − CO), 115 (36, 143 u − CO).

Piperoyl hydroxamic acid

To a solution of N-succinimidyl piperate (1 mmol, 315 mg) in 20 ml dioxane was added 2 ml of redistilled hydroxylamine (22 M) plus 2 ml of water to dissolve a slight turbidity. After incubation for 2 h at 30 °C, the mixture was diluted with water, acidified with HCl (pH 2), and the resulting suspension extracted 6-times with $CHCl_3$. The organic layer was dried ($CaSO_4$), filtered, and evaporated *in vacuo*. The residual oil was crystallized from $CHCl_3$, yielding pale yellow platelets, m.p. 159–160 °C (dec.) in 43% yield. Their purity was confirmed by TLC (systems 1, 6). Elementary analysis gave 62.03% C; 4.72% H; 6.11% N (calcd. for $C_{12}H_{11}NO_4$: 61.80% C; 4.75% H; 6.01% N). MS (70 eV): *m/z* 233 (33%, M^+), 201 (100, M − NHOH), 173 (32, 201 u − CO), 171 (20), 143 (27, 173 u − CH_2O), 115 (83, 143 u − CO).

Piperoyl Coenzyme A

All steps were carried out in the dark to reduce the risk of photo-isomerization at the pentadienoic sidechain of the acyl moiety. CoA (free acid, Boehringer Mannheim; 36 mg, ca 40 μmol) was dissolved in 4 ml 0.1 M $NaHCO_3$. Under constant bubbling of N_2 through this solution, N-succinimidyl piperate (100 μmol, 30 mg) in 6 ml dioxane was added at room temperature in small portions over a period of 45 min, with occasional readjusting the pH with bicarbonate. After standing for further 15 min, completion of the reaction was reached as shown by the "delayed" nitroprusside reaction [6]. The mixture was diluted with water, chilled in ice, acidified with 1 N HCl (pH 1.5), and extracted 4-times with EtOAc. The aqueous phase was passed through a 1 μm membrane filter (Schleicher & Schüll RC-60) and subjected in 0.2 ml portions to isocratic semipreparative HPLC on Merck Lichrosorb RP-18 (5 μm particle size; column 25×0.7 cm; flow rate 3 ml/min; solvent: 48% methanol, 52% 0.01 M NH_4OAc, pH 6.5). The thioester containing fractions were concentrated by rotary-evaporation (30 °C) and lyophilized. Traces of residual salt were eliminated by passage through a small column of Dowex 50 WX8 (H^+, 50–100 mesh) in water. The eluate was lyophilized again, yielding 22 mg (65%) of piperoyl-CoA containing no UV-absorbing impurities as shown by analytical HPLC.

Radioactive piperoyl-CoA was synthesized in scaled-down reaction mixtures following the same strategy. It was found advantageous, however, to omit the isolation of the small amounts of labeled intermediary succinimide ester and to work with the crude reaction mixture from which only the precipitated DCU had been removed by filtration. Under these conditions, recoveries of about 20% with respect to the initial quantity of [^{14}C]piperic acid were observed.

Results and Discussion

Synthesis of piperoyl-CoA

A variety of methods has been developed in the past for the chemical synthesis of acyl-CoA thioes-

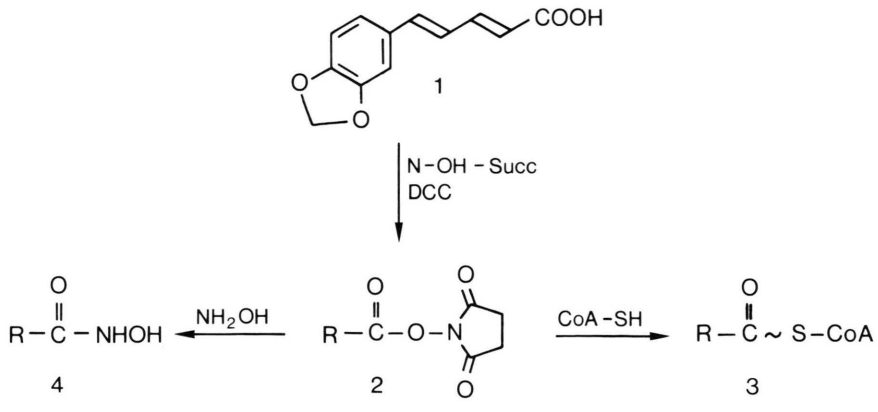

Fig. 1. Scheme for the synthesis of piperoyl-CoA (**3**) and piperoyl hydroxamic acid (**4**) *via* N-succinimidyl piperate (**2**). (**1**) Piperic acid; N-OH-Succ, N-hydroxysuccinimide.

ters. Among these, the route *via* N-succinimidyl esters as activated intermediates proved especially useful. By this technique, for example, the CoA esters of hydroxycinnamic acids [12], tropic acid [13], gallic acid [14], and – quite recently – retinoic acid [7] have been prepared in good yield. This versatile procedure was thus chosen also for the intended synthesis of piperoyl-CoA.

As depicted in Fig. 1, the free acid was converted to the reactive succinimidyl derivative which, in turn, could easily be employed for a subsequent transacylation step with CoA-SH, yielding the desired thioester. For convenience, we prepared a greater quantity of crystalline succinimidyl ester as a readily available and stable stock for the synthesis of piperoyl-CoA. However, as shown for the synthesis of [^{14}C]piperoyl-CoA (*cf.* experimental section), it is not generally necessary to isolate this intermediate, particularly when very small quantities and/or expensive reagents are to be handled.

Rapid and convenient purification of piperoyl-CoA was achieved by reversed-phase HPLC. The isocratic system used by us (*cf.* Experimental) allows to work up comparatively large amounts of material within a few hours. The effectiveness of this method, which certainly supersedes previous column chromatography techniques utilizing DEAE-cellulose [12, 13], sephadex G [14] or LH-20 [15] types, and polyamide [16], is documented by Fig. 2 which illustrates the complete absence of all likely contaminants originating from the synthetic procedure aft-

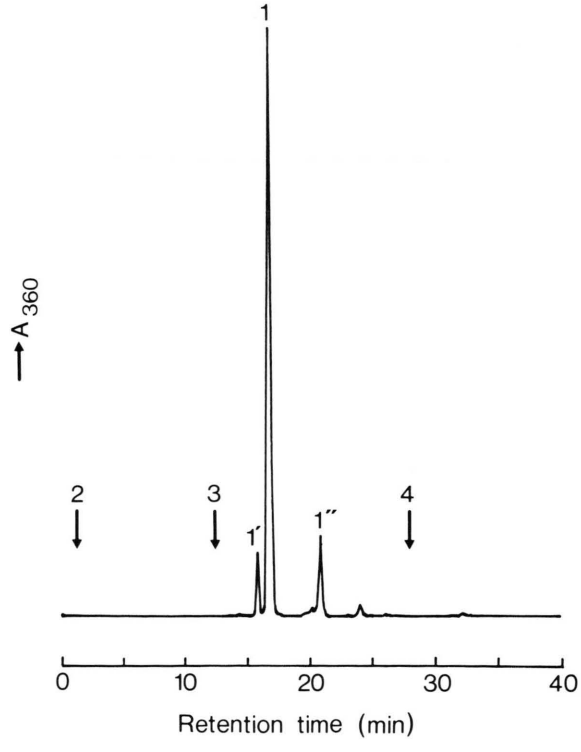

Fig. 2. Analytical HPLC of purified piperoyl-CoA (1). The minor peaks (1′) and (1″) are *cis, trans*-isomers of the pentadienoic side-chain. Arrows indicate the elution position of CoA-SH (2), piperic acid (3), and N-succinimidyl piperate (4) as determined at appropriate wavelengths (*cf.* Table I). Chromatography conditions (*cf.* [7]): Lichrosorb RP-18 (Merck), particle size 5 μm, 180 × 3 mm i.d.; solvent A = methanol, B = 0.01 M NH_4OAc, pH 6.5; linear gradient from 0% A to 90% A within 40 min; flow rate 1 ml/min.

er this HPLC-step. The minor peaks observed are due to inevitable stereoisomers of the piperoyl moiety. Purity of piperoyl-CoA prepared by this means, as determined by UV-spectrometry (see below), usually reached 86–90%, *i.e.* values that are comparable with those of commercially available CoA-esters or free CoA-SH.

Negative results were encountered in an attempt to synthesize piperoyl-CoA according to a more recent method employing intermediary 1-acylimidazole [17]. This fact, together with the reported failure of synthesizing 2-decenoyl-CoA (in contrast to the good yields obtained with other aliphatic acids) [17] and a very recent note on this question [7], clearly indicates the unsuitability of this procedure for the preparation of α,β-unsaturated acyl-CoA esters.

Finally, it should be mentioned that N-succinimidyl piperate served also as an efficient intermediate in the synthesis of piperoyl hydroxamic acid (*cf.* Fig. 1). This compound was required for the quantitative estimation of piperoyl-CoA concentrations (see below) and could not be prepared satisfactorily by the classical route *via* methyl piperate [18].

Properties of piperoyl-CoA

The UV-absorption spectrum of piperoyl-CoA as an important characteristic is depicted in Fig. 3. It shows a maximum at 260 nm due to the adenine moiety of CoA and a second peak in the long-wave UV at 368 nm representing the absorption of the thioester bond. This latter maximum disappears upon cleavage leaving a new peak at 336 nm, the absorption maximum of free piperic acid (*cf.* Table I). The

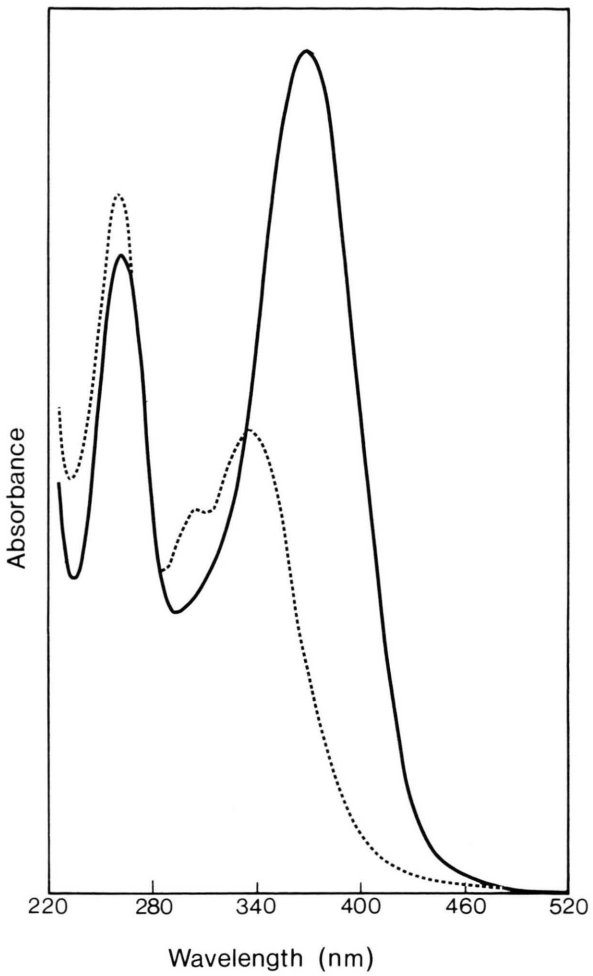

Fig. 3. UV-absorption spectrum of piperoyl-CoA. (—) Spectrum of the thioester; (---) spectrum after hydrolysis in 0.1 N NaOH or 1 M NH_2OH. Both spectra recorded in 0.1 M potassium phosphate buffer, pH 7.0.

Table I. Spectral characteristics of piperoyl coenzyme A and of related piperoyl derivatives.

Compound	Solvent	λ_{max} [nm]	ε [$cm^2\ mol^{-1}$]
Piperic acid	0.1 M K-Pi, pH 7.0	λ_1: 306 (sh)	n.d.
		λ_2: 336	n.d.
N-Succinimidyl piperate	methanol	λ_1: 315 (sh)	n.d.
		λ_2: 360	n.d.
Piperoyl hydroxamic acid, Fe^{3+}-complex	aq. dioxane	540	2.2×10^6
Piperoyl-CoA	0.1 M K-Pi, pH 7.0	λ_1: 260	21.5×10^6
		λ_2: 368	30.8×10^6

K-Pi, potassium phosphate buffer; sh, shoulder; n.d., not determined.

resulting difference spectrum displays a maximum at 376 nm.

As a prerequisite for the calculation of the molar extinction coefficient ε of piperoyl-CoA, the exact concentration of this compound had to be determined. This was done, after hydroxylaminolysis of the thioester, by the hydroxamate assay of Lipmann and Tuttle [5]. Based on the spectral characteristics of the piperoyl hydroxamate-Fe^{3+}-complex (Table I), an ε_{368} of 30.8×10^6 [$cm^2\ mol^{-1}$] was calculated for the thioester linkage. This result was confirmed by comparing the absorbance of [^{14}C]piperoyl-CoA with its specific radioactivity. This exceedingly high ε-value allows the reliable measurement even of minute quantities of piperoyl-CoA; for instance, a concentration of only 1 nmol/ml corresponds to an E_{368} of 0.031. The observed value is in line with an apparent trend, in both qualitative and quantitative terms, regarding the absorption of the thioester bond. Average values of $\varepsilon_{232} \approx 4.5 \times 10^6$ for aliphatic CoA esters [13, 19, 20], $\varepsilon_{272-310} \approx 7 \times 10^6$ for benzoyl-CoA's [14, 21, 22], and $\varepsilon_{311-355} \approx 20 \times 10^6$ [$cm^2\ mol^{-1}$] for cinnamoyl-CoA's [12, 16, 23] have been determined. The data reported here on piperoyl-CoA extends this series, documenting the increasing bathochromy and intensity of the thioester linkage in response to the introduction of additional double bonds.

Piperoyl-CoA was further characterized by determining the rate constants in the presence of alkali or hydroxylamine by photometrically measuring the decrease of the thioester bond [20]. At 25 °C, a half-life $t/2$ of 2.35 min was observed in 0.1 M NaOH which corresponds to an apparent pseudo-first order rate constant k of 0.294 [min^{-1}]. The corresponding value in 1 M NH_2OH was $t/2 = 3.25$ min ($k = 0.213$ [min^{-1}]).

Summarizing the data reported above, it is evident that piperoyl-CoA is easily accessible by chemical means. Its photometric characteristics should provide a convenient and sensitive tool for enzymatic studies on the postulated metabolic role of this thioester.

Acknowledgements

Financial support by the Fonds der Chemischen Industrie is gratefully acknowledged.

[1] T. A. Geissmann and D. H. G. Crout, Organic Chemistry of Secondary Plant Metabolism, p. 170, Freeman, Cooper & Co., San Francisco 1969.
[2] C. R. Bird and T. A. Smith, Phytochemistry **20,** 2345–2346 (1981).
[3] C. R. Bird and T. A. Smith, Phytochemistry **22,** 2401–2403 (1983).
[4] J. Negrel and C. Martin, Phytochemistry **23,** 2797–2801 (1984).
[5] F. Lipmann and L. C. Tuttle, J. Biol. Chem. **159,** 21–28 (1945).
[6] E. R. Stadtman, Methods in Enzymology, **Vol. III,** pp. 931–941, Academic Press, New York 1957.
[7] A. Kutner, B. Renstrom, H. K. Schnoes, and H. F. DeLuca, Proc. Natl. Acad. Sci. USA **83,** 6781–6784 (1986).
[8] R. Fittig and W. H. Mielch, Ann. Chem. Pharm. **152,** 25–58 (1869).
[9] H. Lohaus, J. Prakt. Chem. **119,** 235–271 (1928).
[10] R. B. Loftfield and E. A. Eigner, Biochim. Biophys. Acta **130,** 426–448 (1966).
[11] M. Scholtz, Ber. Deutsch. Chem. Ges. **28,** 1187–1197 (1895).
[12] J. Stöckigt and M. H. Zenk, Z. Naturforsch. **30c,** 352–358 (1975).
[13] G. G. Gross and K. J. Koelen, Z. Naturforsch. **35c,** 363–366 (1980).
[14] G. G. Gross, Z. Naturforsch. **37c,** 778–783 (1982).
[15] J. J. Mieyal, L. T. Webster, and U. S. Siddiqui, J. Biol. Chem. **249,** 2633–2640 (1974).
[16] M. H. Zenk, B. Ulbrich, J. Busse, and J. Stöckigt, Anal. Biochem. **101,** 182–187 (1980).
[17] A. Kawaguchi, T. Yoshimura, and S. Okuda, J. Biochem. **89,** 337–339 (1981).
[18] C. R. Hauser and W. B. Renfrow, in: Organic Syntheses (A. H. Blatt, ed.), **Vol. 2,** pp. 67–68, Wiley & Sons, New York 1963.
[19] R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones (eds.), Data for Biochemical Research, p. 191, Clarendon Press, Oxford 1969.
[20] H. Eggerer and F. Lynen, Biochem. Z. **335,** 540–547 (1962).
[21] D. Schachter and J. V. Taggart, J. Biol. Chem. **203,** 925–934 (1953).
[22] L. T. Webster, J. J. Mieyal, and U. A. Siddiqui, J. Biol. Chem. **249,** 2641–2645 (1974).
[23] G. G. Gross and M. H. Zenk, Z. Naturforsch. **21b,** 683–690 (1966).