Synthesis of Galloyl-Coenzyme A Thioester

Georg G. Gross

Abteilung Allgemeine Botanik, Universität Ulm, Oberer Eselsberg, D-7900 Ulm

Z. Naturforsch. **37 c**, 778–783 (1982); received June 7, 1982

Galloyl-Coenzyme A, 4-O-β-D-Glucosidogalloyl-Coenzyme A, Thioester, 4-O-β-D-Glucosidogalloyl Hydroxamic Acid, N-Succinimidyl 4-O-β-D-Glucosidogallate, Gallotannins

Galloyl-CoA, a potential intermediate in the biosynthesis of gallotannins, has been prepared *via* N-succinimidyl 4-O-β-D-glucosidogallate and 4-O-β-D-glucosidogalloyl-CoA. Besides a major absorption band at 261 nm, the UV-spectra of the purified thioester and its corresponding 4-O-glucoside contain a longer wavelenght absorption band due to the thioester linkage at 305 nm (galloyl-CoA) or at 290 nm (shoulder, glucosidogalloyl-CoA). The molar extinction coefficients ε of the two thioesters were determined *via* the iron-complex of 4-O-β-D-glucosidogalloyl hydroxamic acid; ε_{261}-values of 19.5×10^6 [cm^2 mol^{-1}] and 21.5×10^6 [cm^2 mol^{-1}] were calculated for galloyl-CoA and its glucoside, respectively. Difference spectra, *i.e.* absorbance before esterolysis and after, revealed maximal absorption of the thioester bond at 310 nm ($\Delta\varepsilon = 7.4 \times 10^6$ [cm^2 mol^{-1}]) for galloyl-CoA and at 282 nm ($\Delta\varepsilon = 7.2 \times 10^6$ [cm^2 mol^{-6}]) for glucosidogalloyl-CoA. The two thioesters were further characterized by determining their half-lifes during hydroxylaminolysis and alkaline hydrolysis.

Introduction

Gallotannins are phenolic plant products which consist of a polyol moiety (usually D-glucose) whose hydroxyl groups are esterified wit gallic acid (3,4,5-trihydroxybenzoic acid). The complexity of such esters ranges from the simple β-D-glucogallin (1-galloyl-β-D-glucose) to β-penta-O-galloyl-D-glucose. Esterification at the *m*-hydroxyl of the galloyl residues with further galloyl groups yields polygalloyl esters like the chinese gallotannin from *Rhus semialata*. In contrast to the detailed knowledge of the chemistry and natural distribution of such hydrolyzable tannins (for recent reviews, see [1, 2]), little is yet known concerning the biosynthesis of these compounds. For thermodynamic reasons, the participation of an activated intermediate may by be expected in such esterification reactions. This assumption has been proven by enzymatic studies on the formation of chlorogenic acid (3-O-caffeoyl-D-quinic acid) and related depsides [3–7], acylated flavonols [8] or lupine alkaloids [9]. It has been shown in these investigations that cinnamoyl-CoA thioesters were utilized as carboxyl-activated intermediates. By analogy, it appears conceivable that galloyl-CoA might be involved in the biosynthesis of gallotannins. It should be noted that esters of substituted benzoic acids are common plant constituents, and that their CoA-derivatives have frequently been proposed to function as the required precursors (*cf.* [10]). However, no conclusive data on the biosynthesis of such esters are available. With respect to gallotannins, it was decided to synthesize and characterize the CoA-thioester of gallic acid as a prerequisite for further studies on these questions.

Abbreviations: CoA, CoA-SH, coenzyme A; DCC, dicyclohexyl carbodiimide; DCU, dicyclohexyl urea; PC, paper chromatography; TLC, thin-layer chromatography.

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

0341-0382/82/0900-0778 $ 01.30/0

Experimental

Analytical methods

The chromatography systems used for the identification of galloyl-CoA and of the various gallic acid derivatives required in its synthesis are summarized in Table I. The compounds were detected by inspection under UV-light and further characterized by color reactions. Phenolic hydroxyl groups were detected by spraying with diazotized sulfanilic acid, the presence of vicinal phenolic hydroxyls was demonstrated with 0.5% $FeCl_3$ in methanol. Hydroxamic acids and esters (after pretreatment with 2 M hydroxylamine, pH 6) were stained with acidic $FeCl_3$-solution [11]. Thioesters and free CoA-SH were detected by the "delayed" nitroprusside reaction [12].

Mass spectra were recorded on a Varian MAT 711. C,H,N-analyses were performed by Mikroanalytisches Laboratorium Pascher, Bonn.

Table I. R_f-values of gallic acid derivatives used in the synthesis of galloyl-CoA. I: TLC, cellulose MN 300, *n*-BuOH/HOAc/H_2O = 4:1:5 (upper phase); II: PC, Schleicher & Schüll 2043b, *n*-BuOH/HOAc/H_2O = 20:1:4; III: PC, S & S 2043b, EtOH/0.1 M NaOAc pH 4.5 = 1:1; IV: PC, S & S 2043b, *n*-BuOH/HOAc/H_2O = 5:2:3; V: PC, S & S 2043b, isobutyric acid/NH_3/H_2O = 66:1:33; VI: TLC, polyamide-6, EtOAc/MeCOEt/HCO_2H/H_2O = 5:3:1:1; VII: TLC, Si-gel G, toluene/Et formate/HCO_2H = 5:4:1; VIII: Servacel SA-2 cation exchange paper, 0.05 M K-P_i buffer pH 7.5.

Compound	R_f-value in chromatography system							
	I	II	III	IV	V	VI	VII	VIII
Gallic acid	0.63	0.56	0.66	0.58	0.25	0.55	0.34	0.94
Glucosidogallic acid	0.60	0.59	0.69	0.55	0.30	0.59	0.08	0.94
N-Succinimidyl gallate	0.81	0.43	–	–	–	–	0.28	–
N-Succinimidyl glucosidogallate	0.66	0.68	0.74	0.60	0.42	0.88	0.07	0.36
N-Hydroxysuccinimide	0.57	0.48	0.75	0.59	0.48	0.69	0.20	0.83
Galloyl-CoA	0.33	0.01	0.56	0.18	0.31	0.03	0	–
Glucosidogalloyl-CoA	–	–	0.51	0.12	0.25	–	–	–
Coenzyme A	–	–	0.51	0.19	0.33	–	–	–
Galloyl hydroxamic acid	0.39	0.22	–	–	–	0.33	0.38	–
Glucosidogalloyl hydroxamic acid	0.37	0.22	–	–	–	0.31	0.04	–

Materials

CoA (free acid) and β-glucosidase were obtained from Boehringer Mannheim. Redistilled neutralized hydroxylamine was prepared according to ref. [13]. Ethyl gallate (Fluka) was purified by recrystallization from water. Glucosidogallic acid (4-O-β-D-glucopyranosylgallic acid) was synthesized by reacting ethyl gallate with α-acetobromoglucose (Serva Heidelberg), yielding ethyl tetraacetylglucosidogallate, and subsequent removal of the protecting groups by alkaline hydrolysis [14]. The product was pure as judged by chromatography (solvents I, VI, VII), stained positively with diazotized sulfanilic acid, but not with $FeCl_3$, and gave pure gallic acid after treatment with β-glucosidase.

Chemical syntheses

N-Succinimidyl 4-O-β-D-glucosidogallate

This ester was prepared analogously to the synthesis of succinimidyl myristate [15]. Glucosidogallic acid (4 mmol, 1.32 g) and N-hydroxysuccinimide (4 mmol, 460 mg) were dissolved by refluxing in dioxane. After cooling to room temperature, DCC (4 mmol, 824 mg) was added in small portions under stirring. The mixture was kept overnight and then the DCU was filtered off and the solvent removed by rotary-evaporation. The resulting solid was purified at 4 °C in 0.3–0.5 g portions by passage through a Sephadex G-10 column (2.5 cm i.d. × 30 cm) with water as eluant. The fractions containing the ester (V_e/V_0 = 5.2) were pooled and lyophilized (yield 56%). The product was analyzed chromatographically (systems VII, VIII); it gave no color reaction with $FeCl_3$ alone, but stained after treatment with hydroxylamine/$FeCl_3$ or diazotized sulfanilic acid.

N-Succinimidyl gallate

N-Succinimidyl glucosidogallate (12.5 µmol, 5.25 mg) was dissolved in 0.3 ml 0.1 M sodium acetate buffer, pH 5.0, and β-glucosidase (10 U) was added. After incubation under N_2 for 30 min at 37 °C, the incubation mixture was chilled in ice, brought to pH 3 with 1 N HCl, and thoroughly extracted with ether. The organic phase was evaporated and the product was analyzed chromatographically (systems I, II, VII). It stained with $FeCl_3$ and diazotized sulfanilic acid. The presumed structure was further confirmed by FD-MS: *m/e* 267 (M^+).

4-O-β-D-Glucosidogalloyl hydroxamic acid

N-Succinimidyl glucosidogallate (480 µmol, 203 mg) and redistilled hydroxylamine (4 mol) in a total volume of 2 ml were incubated for 1.5 h at 30 °C. The reaction mixture was then chilled in ice and purified by gel-filtration as described above. The fractions containing the hydroxamic acid (V_e/V_0 = 3.2) were combined and lyophilized (yield 38%). The product showed a single spot after chromatography (solvents I, II, VI, VII) which gave the expected color reactions with $FeCl_3$ or diazotized sulfanilic

acid. Elementrary analysis gave 43.23% C; 5.13% H; 4.10% N (calcd. for $C_{13}H_{17}NO_{10}$: 44.96% C; 4.94% H; 4.03% N).

Galloyl hydroxamic acid

Galloyl hydroxamic acid was prepared from the corresponding glucoside as described above for N-succinimidyl gallate. The product was pure as shown by chromatography (systems I, VII) and gave the expected color reactions. Its structure was further proven by EI-MS (70 eV): *m/e* 185 (M^+), 153 ($C_6H_2(OH)_3CO^+$).

4-O-β-D-Glucosidogalloyl-CoA

CoA (18 mg) was dissolved in 2.0 ml of ice-cold water and 0.2 ml 1 M $NaHCO_3$ was added. Under constant bubbling of N_2 through this solution, N-succinimidyl glucosidogallate (50 μmol, 21.4 mg) was added in small portions over a period of 45 min, with occasional readjusting the pH of the mixture with bicarbonate. After standing on ice for further 15 min, the solution was applied to a column (2.5 cm i.d. × 30 cm) of Sephadex G-10 in water. The fractions containing the thioester ($V_e/V_0 = 1$) were pooled and lyophilized (yield 50–70%). Chromatographical analysis (systems III–V) revealed that the product still contained a few percent of impurities, but that it was free of N-hydroxysuccinimide, glucosidogallic acid and N-succinimidyl glucosidogallate as the most likely contaminants.

Galloyl-CoA

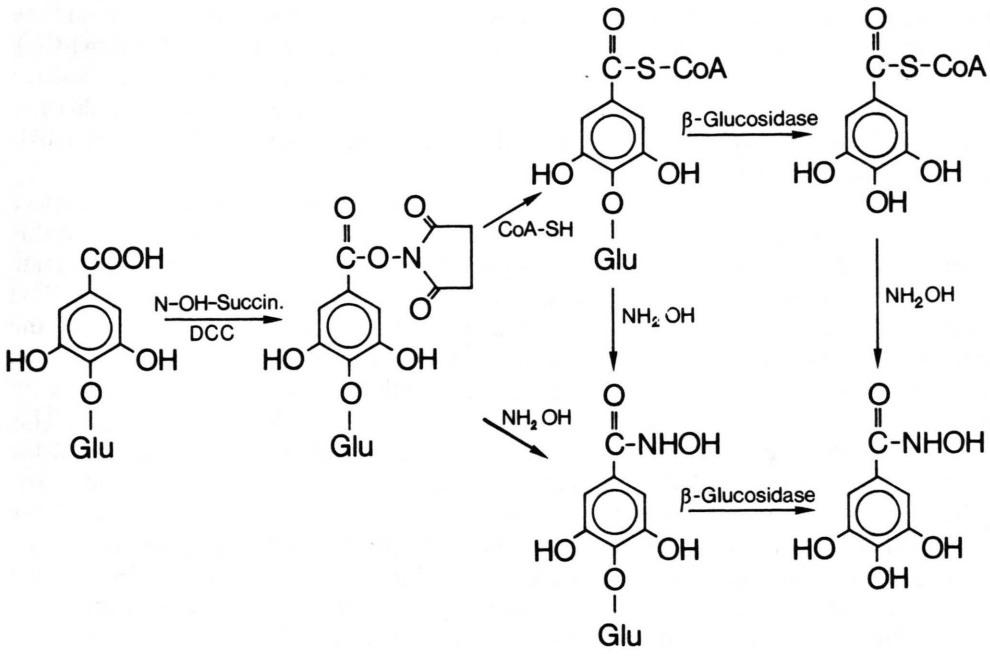
Glucosidogalloyl-CoA (20 mg) and β-glucosidase (10 U) were dissolved in 2.0 ml 0.1 N sodium acetate buffer, pH 5.0, and incubated under N_2 for 30 min at 37 °C. The mixture was then chilled in ice, filtered to remove a slight turbidity, and subjected to gel-filtration on a Sephadex G-25 (coarse) column (1.5 cm i.d. × 20 cm) in order to remove the enzyme. Two peaks were observed; the second one ($V_e/V_0 = 2.0$) gave the color reactions characteristic of the desired thioester. These fractions were pooled, lyophilized, redissolved in 1.5 ml ice-cold water and rechromatographed on Sephadex G-10 (experimental conditions as above). Again, two peaks emerged from the column of which the second one ($V_e/V_0 = 1.5$) was identified as galloyl-CoA. The purest fractions as judged by chromatography (systems III–V) and color reactions were pooled, lyophilized and stored in the desiccator at −20 °C (yield 40–60%).

Results and Discussion

Synthesis of galloyl-CoA

Besides enzymatic methods, a variety of chemical procedures is available for the synthesis of acyl-CoA thioesters. Acid anhydrides [16], acid chlorides [17], mixed anhydrides [18, 19] or acylimidazole [20] have been used as acylating reagents. These methods, however, frequently fail in those cases where the acyl moiety bears reactive functional groups – a situation which certainly applies to the intended synthesis of galloyl-CoA. Similar difficultis have previously been encountered in the preparation of cinnamoyl-CoA esters, and this problem was solved by the use of thiophenol or N-succinimidyl esters [21]. As the latter derivative could be employed successfully by us also for the synthesis of tropoyl-CoA [22], we decided to use this method for the preparation of galloyl-CoA, too. Preliminary experiments, however, indicated that the direct esterification of gallic acid and N-hydroxysuccinimide was impracticable because of the formation of multiple degradation products and/or by-products. This problem could be avoided by the use of 4-O-glucosidogallic acid instead of the free acid (*cf.* [21]). This had also the important advantage that, due to the absence of vicinal phenolic OH-groups, the hydroxamate assay of Lipmann and Tuttle [11] could be employed for the quantitative determination of the intermediate products synthesized in this investigation. The entire reaction sequence which finally enabled us to prepare galloyl-CoA is summarized in Scheme I.

N-Succinimidyl glucosidogallate, the first intermediate along this route, could be synthesized in good yields by the use of DCC. Problems were encountered only in the purification of the crude product. After negative results with DEAE-cellulose, Dowex 1X4 and Sephadex LH-20 under various conditions, it was found that gel-filtration on Sephadex G-10 in water provided an efficient and convenient method to eliminate the contaminating starting-reagents and by-products. The identity of the pure ester was proven by means of chromatography and group-specific color reactions, as well as



Scheme I. Reactions involved in the synthesis and characterization of galloyl-CoA. Glu = glucosyl-residue.

by chemical derivatization. Hydroxylaminolysis afforded the corresponding hydroxamic acid which, in turn, gave galloyl hydroxamic acid after treatment with β-glucosidase. Direct enzymatic hydrolysis of the ester gave the expected aglycone N-succinimidyl gallate.

Glucosidogalloyl-CoA was prepared by transacylation of the corresponding succinimide ester with CoA-SH in slightly alkaline medium. The purified thioester was characterized by two ways. First, alkaline hydrolysis, followed by treatment with β-glucosidase, resulted in the formation of free gallic

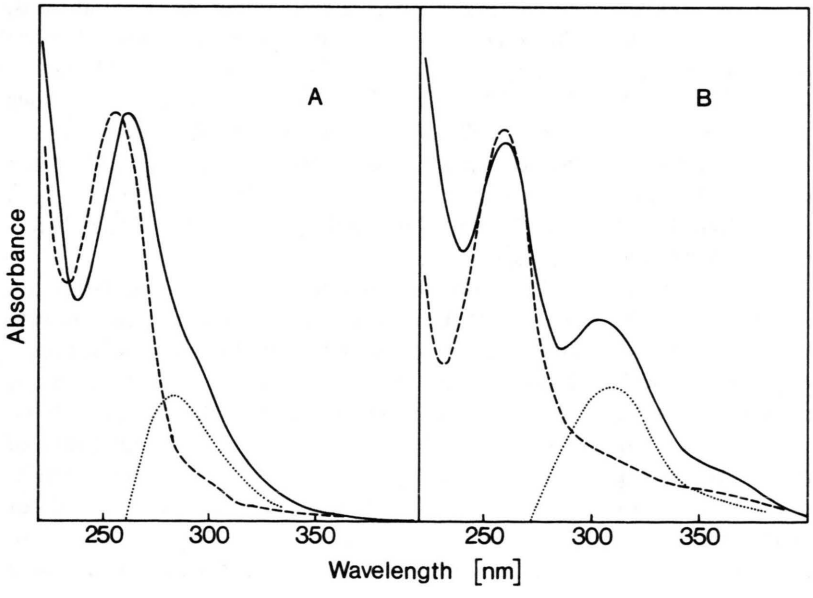


Fig. 1. UV-spectra of 4-O-β-D-glucosidogalloyl-CoA (A) and galloyl-CoA (B). (——) Spectrum of the thioester; (----) spectrum after hydrolysis in 0.1 N NaOH, or hydroxylaminolysis in 1 M NH_2OH, pH 6; (....) difference spectrum. All spectra recorded in 0.1 M potassium phosphate buffer, pH 7.0.

acid. Second, hydroxylaminolysis gave a product which was indistinguishable from glucosidogalloyl hydroxamic acid prepared directly from the common precursor N-succinimidyl glucosidogallate. Further evidence was obtained by the typical color reactions with diazotized sulfanilic acid and hydroxylamine/$FeCl_3$. The nitroprusside reaction, in contrast, resulted in an uncharacteristic, faint, and very slowly developing greyblue color. A possible explanation for this unusual behavior may be found in the exceedingly long half-life observed for this thioester in alkaline solution (see below).

Such problems were not encountered with galloyl-CoA, the final product, which was isolated after enzymatic removal of the protecting glucosyl residue. Treatment with nitroprusside and alkali resulted in the immediate appearance of a brick-red color which, however, could clearly be distinguished from the red color obtained with free CoA-SH. The nature of this thioester was further confirmed by alkaline hydrolysis, yielding gallic acid, and by hydroxylaminolysis which led to the formation of a product that cochromatographed with authentic galloyl hydroxamic acid in several solvents.

Characteristics of glucosidogalloyl-CoA and galloyl-CoA

The UV-spectra of galloyl-CoA and its 4-O-glucoside are depicted in Fig. 1. Both spectra are characterized by a major maximum at 261 nm due to the adenine moiety of CoA. The thioester linkage causes additional light-absorbance at longer wavelenghts, namely a shoulder around 290 nm (glucosidogalloyl-CoA) or a second peak at 305 nm (galloyl-CoA), which drastically decreases upon hydroxylaminolysis or alkaline hydrolysis. The resulting difference spectra had maxima at 282 nm and 310 nm, respectively. Moreover, it was found that the spectrum on an equimolar mixture of CoA and glucosidogallic acid was identical with that of the corresponding thioester after esterolysis. The observed spectral characteristics are in close agreement with those reported previously for benzoyl-CoA [23], its mono-hydroxylated derivatives [24] or veratroyl-CoA (3,4-dimethoxybenzoyl-CoA) [25]. Comparison of these spectra reveals that increasing substitution of the benzoyl moiety shifts the maximum of the thioester linkage to longer wavelengths as it is already known for the analogous cinnamoyl derivatives [25, 26]. In this context, it should be noted that the spectral properties of galloyl-CoA provide the basis for a convenient and sensitive photometric assay in enzymatic studies, as this has been widely used already for the related cinnamoyl-CoA esters.

The next problem studied was the quantitative determination of the CoA-esters synthesized in this investigation. This was done *via* the hydroxamate assay [11]. With analytically pure glucosidogalloyl hydroxamic acid, an absorption maximum of the hydroxamate-iron complex at 530 nm was recorded and a molar extinction coefficient ε of 1.52×10^6 [$cm^2\,mol^{-1}$] was calculated for this wavelenght. This value is in good accordance with those found for benzohydroxamic acid (1.56×10^6) or cinnamoyl hydroxamic acid (1.68×10^6, [21]). Using the above value for the quantification of glucosidogalloyl-CoA, we found and ε_{261} of 21.5×10^6 [$cm^2\,mol^{-1}$] and a $\Delta\varepsilon_{282}$ of 7.2×10^6 [$cm^2\,mol^{-1}$] for the maximum of the difference spectrum.

The corresponding data for galloyl-CoA could not be determined directly by this means since the phenolic OH-groups of gallic acid interfere with the hydroxamate assay. We therefore measured the exact concentration of a sample of glucosidogalloyl-CoA by UV-spectrometry, hydrolyzed quantitatively with β-glucosidase, readjusted to pH 7.0, and recorded the UV-spectrum of the liberated galloyl-CoA (the absorbance of the enzyme within the solution contributed to less than 1% of the total extinction and could thus be neglected). Under these conditions, the following molar extinction coefficients were calculated for galloyl-CoA: $\varepsilon_{261} = 19.5 \times 10^6$; $\varepsilon_{305} = 10.1 \times 10^6$; $\Delta\varepsilon_{310} = 7.4 \times 10^6$ [$cm^2\,mol^{-1}$]. These values agree well with those published previously for benzoyl and hydroxybenzoyl-CoA's [23, 24] which were in the range of $21.1-21.4 \times 10^6$ for ε_{261} and $6.4-7.2 \times 10^6$ [$cm^2\,mol^{-1}$] for the $\Delta\varepsilon$ of the difference spectra.

The thioesters synthesized in this investigation were further characterized by determining the rate constants in the presence of hydroxylamine or alkali. Hydroxylaminolysis was carried out at 30 °C in 1 M NH_2OH, pH 6; the decrease of the thioester bond was measured photometrically at the maximum of the difference spectrum [27]. Under these conditions, a half-life $t/2$ of 1.55 min was observed for glucosidogalloyl-CoA, which corresponds to an apparent pseudo-first order rate constant k of 0.431

[min^{-1}]. The analogous value for galloyl-CoA was $t/2 = 6.6$ min ($k = 0.103$ [min^{-1}]).

Determination of the esterolysis constants in alkali was complicated by the presence of ionizable hydroxyl-groups in the CoA-esters which causes a pronounced change of the UV-spectra at increasing OH^--concentrations, thus preventing a continuous photometric assay. Aliquots of a solution of glucosidogalloyl-CoA in 0.1 N NaOH were therefore neutralized in 5 min-intervals, buffered with potassium phosphate, pH 7.0, and analyzed at 282 nm. Under these conditions, a $t/2$ of 28 min ($k = 0.023$ [min^{-1}]) was determined. The correctness of this unusually long half-life was proven by determining the amount of hydrolytically released CoA-SH using 5,5′-dithiobis (2-nitrobenzoic acid) [28]; exactly the same values as above were obtained by this procedure. With respect to galloyl-CoA, neither of these methods could be employed successfully. It appears that the galloyl-moiety with its extremely troublesome 3,4,5-trihydroxy configuration makes such studies virtually impossible.

Summarizing the data reported above, it is evident that CoA-esters can be synthesized also with such labile compounds like gallic acid in satisfying yield and purity. The latter, as determined by UV-spectrometry, was usually between 83 and 98%, *i.e.* a value which is equivalent to the purity of commercially available CoA-esters or free CoA-SH. Moreover, the galloyl-thioesters appear to be fairly stable; no significant degradation was observed with samples that had been stored desiccated at $-20°$ for several months. Thus, a basis has been provided now for attacking the question whether galloyl-CoA is involved in the biosynthesis of gallotannins, a problem which is presently under investigation in our laboratory.

Acknowledgements

I am indebted to Prof. H. Eggerer, Dr. J. Stöckigt, and Prof. M. H. Zenk, München, for helpful suggestions, to Dr. G. Schmidtberg, Ulm, for recording and interpretation of mass-spectra, and to Mrs. A. Müller for excellent technical assistance.

[1] E. Haslam, Biochemistry of Plant Phenolics (T. Swain, J. B. Harborne, and C. F. van Sumere, eds.), Rec. Adv. Phytochem. **Vol. 12,** p. 475, Plenum Press, New York, London 1979.
[2] E. Haslam, The Biochemistry of Plants. **Vol. 7,** Secondary Plant Products (E. E. Conn, ed.), p. 527, Academic Press, New York 1981.
[3] J. Stöckigt and M. H. Zenk, FEBS Lett. **42,** 131 (1974).
[4] B. Ulbrich and M. H. Zenk, Phytochemistry **18,** 929 (1979).
[5] B. Ulbrich and M. H. Zenk, Phytochemistry **19,** 1625 (1980).
[6] M. J. C. Rhodes and L. S. C. Wooltorton, Phytochemistry **15,** 947 (1976).
[7] M. J. C. Rhodes, L. S. C. Wooltorton, and E. J. Lourenço, Phytochemistry **18,** 1125 (1979).
[8] M. H. Saylor and R. L. Mansell, Z. Naturforsch. **32 c,** 765 (1977).
[9] I. Murakoshi, M. Ogawa, K. Toriizuka, J. Haginiwa, S. Ohmiya, and H. Otomasu, Chem. Pharm. Bull. **25,** 527 (1977).
[10] G. G. Gross, The Biochemistry of Plants, **Vol. 7.** Secondary Plant Products (E. E. Conn, ed.), p. 301, Academic Press, New York 1981.
[11] F. Lipmann and L. C. Tuttle, J. Biol. Chem. **159,** 21 (1945).
[12] E. R. Stadtman, Methods in Enzymology, **Vol. III,** p. 931, Academic Press, New York 1957.
[13] R. B. Loftfield and E. A. Eigner, Biochim. Biophys. Acta **130,** 426 (1966).
[14] E. Fischer and H. Strauß, Ber. Deutsch. Chem. Ges. **45,** 3773 (1912).
[15] A. Paquet, Can. J. Chem. **57,** 2775 (1979).
[16] E. J. Simon and D. Shemin, J. Amer. Chem. Soc. **75,** 2520 (1953).
[17] W. Seubert, Biochem. Prep. **7,** 80 (1960).
[18] T. Wieland and L. Rueff, Angew. Chem. **65,** 186 (1953).
[19] Y. Kawanami, Y. Dainobu, J. Inanaga, T. Katsuki, and M. Yamaguchi, Bull. Chem. Soc. Jpn. **54,** 943 (1981).
[20] A. Kawaguchi, T. Yoshimura, and S. Okuda, J. Biochem. **89,** 337 (1981).
[21] J. Stöckigt and M. H. Zenk, Z. Naturforsch. **30 c,** 352 (1975).
[22] G. G. Gross and K. J. Koelen, Z. Naturforsch. **35 c,** 363 (1980).
[23] D. Schachter and J. V. Taggart, J. Biol. Chem. **203,** 925 (1953).
[24] L. T. Webster, J. J. Mieyal, and U. A. Siddiqui, J. Biol. Chem. **249,** 2641 (1974).
[25] M. H. Zenk and G. G. Gross, Z. Pflanzenphysiol. **53,** 356 (1965).
[26] G. G. Gross and M. H. Zenk, Z. Naturforsch. **21 b,** 683 (1966).
[27] H. Eggerer and F. Lynen, Biochem. Z. **335,** 540 (1962).
[28] G. L. Ellman, Arch. Biochem. Biophys. **82,** 70 (1959).