Chemical Synthesis of Tropoyl Coenzyme A

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Tropoyl coenzyme A has been synthesized in good yields via the corresponding n-hydroxysuccinimide ester. The UV-spectrum of the purified thioester has an absorption maximum at 257 nm; at this wavelength, a molar extinction coefficient of \(19.2 \times 10^3 \text{[cm}^{-1} \text{mol}^{-1}]\) has been determined. Upon alkaline hydrolysis of the thioester bond a difference spectrum with \(\Delta \varepsilon_{235} = 4.8 \times 10^3 \text{[cm}^{-1} \text{mol}^{-1}]\) has been observed. Attempts to prepare 2-phenylmalonyl coenzyme A by the same technique gave negative results.

Introduction

Hyoscyamine and scopoline are the most common tropane alkaloids found in the Solanaceae. These compounds represent esters of tropine or scopine as the basic moiety and L-tropic acid as the acidic component, whereas racemic D,L-tropic acid is obtained after hydrolysis of atropine. The results from feeding experiments with sterile root cultures (for references see [1]) or tissue cultures [2–4] of Datura suggested that esterification of a tropane derivative with tropic acid represents the final step in the biosynthesis of these alkaloids. In biochemical studies an atropine esterase has been discussed to be involved in this reaction (cf. [1]). For thermodynamic reasons, however, the participation of an activated intermediate must be postulated in such a conversion. By analogy to the now well understood biosynthesis of various cinnamic acid esters (cf. [5]), the CoA-derivative of tropic acid would be a likely candidate for this intermediate. This view is supported by a short note on the \textit{in vitro} synthesis of hyoscyamine in the presence of ATP and CoA [6]. However, no details of this reaction have been published to date. We thus decided to synthesize and characterize tropoyl-CoA as a prerequisite for further studies on these questions.

Experimental

Methyl tropate was prepared by refluxing D,L-tropic acid in methanol and crystallized from ethyl light petrol (m. p. 38–39 °C; lit. [7] 36–37.5 °C). Monoethyl 2-phenylmalonate was obtained by partial hydrolysis of the diethyl ester [8]; m. p. 77–78 °C (lit. 76–77 °C). Identity and purity of these esters were confirmed by C, H-analyses and thin-layer chromatography (silica gel; solvent I: toluene: ethyl formate : formic acid = 5 : 4 : 1; II: chloroform : benzene : ethyl methyl ketone = 7 : 2 : 1; III: ethanol : water : ammonia = 78 : 9.5 : 12.5).

\textit{[Carboxyl-}^{14}\text{C]}\text{tropic acid was synthesized from [carboxyl-}^{14}\text{C]}\text{phenylacetic acid (CEA, Gif-sur-Yvette) with a specific activity of 10.75 \mu\text{Ci}/\text{mmol} [9]. The crystallized product (m. p. 115 °C; lit. [10] 115–116 °C) was pure as judged by chromatography in solvents I and II. Tropoyl N-hydroxysuccinimide ester was prepared analogous to the synthesis of the corresponding cinnamoyl derivatives [11]. The crude product was crystallized twice from benzene (yield 50%). The pure ester (m. p. 113–116 °C) showed a single spot after thin-layer chromatography (silica gel; solvent IV: chloroform : methanol = 20 : 1; V: chloroform : ethyl acetate : benzene : ethyl methyl ketone : light petrol = 7 : 1 : 2 : 3 : 3) and stained positively after treatment with hydroxylamine and FeCl₃. Analysis gave 59.33% C; 5.12% H; 5.34% N (calcd. for C₁₃H₁₃NO₅: 59.31% C; 4.98% H; 5.32% N). Phenylacetyl N-hydroxysuccinimide ester was prepared analogously.

Tropoyl-CoA was prepared by transesterification of tropoyl N-hydroxysuccinimide with CoA [11]. In preliminary experiments, purification of the crude product was achieved by paper chromatography (solvent VI: isobutyric acid : ammonia : water = 66 : 1 : 33; VII: n-butanol : acetic acid : water = 5 : 2 : 3; VIII: ethanol : 0.1 N sodium acetate pH 4.5 = 1 : 1). Yields, based on the initial amount of free

Abbreviations: CoA, CoA-SH, coenzyme A; DCC, dicyclohexyl carbodiimide.

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CoA-SH as determined with the phosphotrans-acetylase assay [12], reached 46%. Larger quantities of tropoyl-CoA were purified at 4 °C on DEAE-cellulose columns using a linear sodium formate gradient [13]. The tropoyl-CoA containing fractions were pooled, desalted by passage through Dowex 50 W-X 8 (H+-form) [13] and lyophilized. Over-all recoveries of the product prepared by this procedure were between 35 and 45% with respect to CoA.

Radioactive tropoyl-CoA could be synthesized following the same strategy via purified [carboxyl-14C]tropoyl N-hydroxysuccinimide. We found it more convenient and also more economic, however, to omit the isolation of the labeled intermediary succinimide ester and to add CoA directly into the crude reaction mixture from which only the precipitated dicyclohexyl urea had been removed by filtration. Under these conditions, recoveries of about 15% with respect to the initial amount of labeled tropic acid and about 30% with respect to CoA were observed.

Tropoyl hydroxamic acid was synthesized from methyl tropate as described for benzoyl hydroxamic acid [14]. The crystalline free acid (yield 41%) had a m.p. of 167–169 °C; elementary analysis gave 59.64% C; 6.13% H; 7.73% N (calcd. for C9H10N03: 59.66% C; 6.12% H; 7.73% N). Phenylacetyl hydroxamic acid was prepared analogously (m. p. 123–127 °C). Purity of hydroxamic acids was confirmed by paper chromatography (solvents VI–VIII); larger amounts from preparations containing up to 100 mg CoA were purified by DEAE-cellulose column chromatography (cf. Experimental).

The identity of tropoyl-CoA was checked by several methods. After paper chromatography, the thioester gave the characteristic “delayed” color reaction upon treatment with nitroprusside reagent under alkaline conditions [15]. Spraying with neutralized hydroxylamine, followed by FeCl3, resulted in the development of the typical brown-violet color. The existence of an activated tropoyl derivative was further proven by hydroxylaminolysis of free tropoyl-CoA in 1 M hydroxylamine at pH 7 and subsequent chromatographical comparison of the reaction product with authentic tropoyl hydroxamic acid.

When the UV-spectrum of tropoyl-CoA was recorded, a single absorption maximum at 257 nm was observed which corresponds to the adenine moiety of CoA (Fig. 1). This peak remained unaltered after alkaline hydrolysis, a decrease in absorbance, however, occurred at shorter wavelengths. The difference spectrum had a maximum at 235 nm, indicating the presence of an aliphatic thioester bond. The molar extinction coefficient ε of tropoyl-CoA was determined by several methods. First, the absorbance of [14C]tropoyl-CoA was compared with its specific ra-
dioactivity. As an average from several determinations, an $e_{235}$ of $20.2 \times 10^6$ [cm$^2$ mol$^{-1}$] was calculated. Second, we determined the concentration of tropoyl-CoA by the hydroxamate assay of Lipmann and Tuttle [16]. With analytically pure tropoyl hydroxamic acid, an absorption maximum of the hydroxamate-iron complex at 512 nm was recorded and an $e$ of $1.18 \times 10^6$ [cm$^2$ mol$^{-1}$] was calculated for this wavelength. Using this value for the quantification of tropoyl-CoA, we found an $e_{235}$ of $18.2 \times 10^6$ [cm$^2$ mol$^{-1}$]. A third series of experiments was based on the enzymatic analysis of tropoyl-CoA with $\beta$-hydroxyacyl-CoA dehydrogenase [12], resulting in an $e_{257}$ of $23.3 \times 10^6$[cm$^2$ mol$^{-1}$]. Most probably, this value is somewhat too high if one considers the risk of incomplete conversions in this comparatively complex assay. As an average from the first two determinations we propose an $e_{257}$ of $19.2 \times 10^6$ [cm$^2$ mol$^{-1}$] for tropoyl-CoA. Using this value, one can calculate an $e_{235}$ of $11.5 \times 10^6$ [cm$^2$ mol$^{-1}$] for the maximum of the difference spectrum and an $\Delta e_{235}$ of $4.8 \times 10^6$ [cm$^2$ mol$^{-1}$] upon hydrolysis of the thioester bond. These data are in good accordance with those previously published for related compounds (cf. [15, 17, 18]).

The foregoing results clearly demonstrate the facile synthesis of tropoyl-CoA via the corresponding N-hydroxysuccinimide ester. In further experiments, using the same technique, we attempted the synthesis of 2-phenylmalonyl-CoA which has occasionally been discussed as a potential intermediate in the biosynthesis of tropic acid [19, 20]. Elementary analysis of the crystals obtained after esterification of 2-phenylmalonic acid and N-hydroxysuccinimide did not give the expected values which, however, agreed well with those calculated for the corresponding phenylacetyl derivative. This result was confirmed by comparing the reaction product with pure phenylacetyl N-hydroxysuccinimide ester; melting points (116—118 °C) and $R_f$-values in solvents I and II were identical for both substances. The same situation was observed in attempts to prepare 2-phenylmalonyl monohydroxamic acid from monoethyl phenylmalonate. The isolated product was clearly shown to be identical with phenylacetyl hydroxamic acid by means of C,H,N-analysis and determination of the melting points. From these results it is evident that the pronounced lability of phenylmalonic acid [21] poses at least extreme difficulties for the synthesis of carboxyl-activated derivatives of this dicarboxylic acid.

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