Synthesis, Analysis and Characterization of the Coenzyme A Esters of o-Succinylbenzoic Acid, an Intermediate in Vitamin K₂ (Menaquinone) Biosynthesis

Rainer Kolkmann and Eckhard Leistner

Institut für Pharmazeutische Biologie, Rheinische Friedrich-Wilhelms-Universität, D-5300 Bonn, Bundesrepublik Deutschland


Introduction

Recent advances in the understanding of vitamin K₂ biosynthesis revealed that the reaction initiating its biosynthesis starts with iso-chromic acid [1], proceeds via 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylic acid [2] and yields o-succinylbenzoic acid (Fig. 1, 1) which is activated to give a coenzyme A ester [3] assuming that the “free” carboxyl group would react with the enzymically formed coenzyme A ester [3] assuming that the “free” carboxyl group would react with the methylating reagent. Mild hydrolysis of the resulting product gave the “aliphatic” methyl ester of vitamin K₂, Biosynthesis

One of the carboxyl groups of o-succinylbenzoic acid (OSB, 1) is activated during the biosynthesis of vitamin K₂ and anthraquinones. In an attempt to determine the exact structure of the naturally occurring OSB coenzyme A thioester, the three possible esters were prepared via the imidazolides of OSB. HPLC systems were developed for the successful separation of the esters and their structural analogues. The structures of the coenzyme A esters were investigated and their products of hydrolysis determined quantitatively. Two of the esters were highly labile at pH 7 and 30 °C. UV spectroscopy is a suitable method to distinguish between different positions of activation. Fast atom bombardment mass spectrometry was successfully employed to characterize two of the esters.

The data strongly indicate that the “aliphatic” coenzyme A ester is the intermediate in vitamin K₂ and anthraquinone biosynthesis.

Abbreviations: ADP, adenosine-di-phosphate; AMP, adenosine-mono-phosphate; CDB, 1,1’-carbonyldiimidazole; CoA, CoASH, coenzyme A; DHNA, 1,4-dihydroxy-2-naphthoic acid; DMSO-d₆, deuterated dimethylsulfoxide; FAB, fast atom bombardment; HPLC, high performance liquid chromatography; OSB, o-succinylbenzoic acid (i.e. menaquinone) in microorganisms [6, 7] or vitamin K₂ (i.e. phylloquinone) in chloroplasts of higher plants [8, 9]. A similar pathway leading to vitamin K₁ in a cell suspension culture of Catalpa ovata has been proposed [10].

The first aromatic intermediate in vitamin K biosynthesis is o-succinylbenzoic acid (1). This compound is activated in cell-free extracts of Escherichia coli and Mycobacterium phlei to give a mono-coenzyme A ester [4]. Since o-succinylbenzoic acid has two carboxyl groups, the question arises which of the two functional groups is activated. In an attempt to answer this question we methylated (diazomethane) the enzymically formed coenzyme A ester [3] assuming that the “free” carboxyl group would react with the methylating reagent. Mild hydrolysis of the resulting product gave the “aliphatic” methyl ester of...
o-succinyl benzoic acid [11]. This led to the conclusion that the “aromatic” carboxyl group was activated in the mono-coenzyme A ester, a finding which was in agreement with previous assumptions [4, 6]. It was desirable to corroborate this result and to synthesize the coenzyme A esters (Fig. 1, 5, 6, 7) of o-succinylbenzoic acid (1). The experimental approach and characterization of the thiol esters, including spectroscopic and analytical data, are described in the present communication. A preliminary account of part of this investigation has been published [12].

Materials and Methods

**FAB mass spectrometry** was performed on a VG analytical ZAB-HF reversed geometry mass spectrometer with xenon as collision gas. Samples were dissolved in MeOH (5 µg/µl), 1–2 µl of the solution was applied to the stainless steel target and mixed with 2 µl of thioglycerol (i.e. 1-mercapto-2,3-propanediol).

Spectra were recorded at 7 kV acceleration voltage, registering the negative or positive FAB ions, respectively. Mass peaks of thioglycerol (matrix) were subtracted. Hence, spectra depicted in Fig. 10 and 11 show essentially mass peaks of thiol esters or their fragments.

For FAB mass spectrometry, the CoA esters were purified by HPLC with the H₂PO₄ containing eluent and separated from H₂PO₄ in a second HPLC run with HOAc (0.1 m) in MeOH–H₂O (30:70), followed by evaporation and freeze-drying.

The PC and TLC systems used for identification and separation of OSB derivatives are given in Table I. The compounds were detected by inspection under UV light and, where possible, by an additional colour reaction with nitroprusside reagent [13].

Chemical syntheses

Unlabelled 4-(2’-carboxyphenyl)-4-oxobutanoic acid (OSB) (1) and its spirilactone (11) were synthesized following the method of Roser [14] with slight modifications [15]. [1-¹⁴C]OSB (1) (0.26 MBq/µmol) was prepared according to [16] using sodium [1-¹⁴C]pyruvate (0.85 GBq/mmol, Amersham Buchler, Braunschweig, FRG).

The “aromatic” methyl ester (i.e. 4-(2’-methoxy-carbonylphenyl)-4-oxobutyrate) (3) of OSB (1) was synthesized as described previously [11]; the complete spectroscopic data are as follows:

**UV**: MeOH λ_max nm (logε): 280sh (3.02), 276 (3.04), 230 (3.90), 210 (4.05); IR: KBr v_max cm⁻¹: 2960, 2940, 2600, 1715, 1600, 1500, 1440, 770; ¹H NMR (90 MHz, CDCl₃): δ 2.77–2.91 (2H, m, H-2), 3.09–3.26 (2H, m, H-3), 3.88 (3H, s, 2’-COOCH₃), 7.45–7.92 (4H, m, H-3’–H-6’), 9.50 (1H, broad s, 1-COOH). EI-MS 70 eV, m/z (rel. int.): 236 [M]⁺ (0.3), 221 [M–CH₃]⁺ (1.3), 205 [M–OCH₃]⁺ (4), 163 [M–CH₂COOCH₃]⁺ (100). The “aliphatic” methyl ester (i.e. methyl 4-(2’-carboxyphenyl)-4-oxobutyrate) (2) of OSB (1) was obtained by alkaline hydrolysis (0.1 m methanolic NaOH, 10 min, 20 °C) of the OSB dimethyl ester (4) [11].

**UV**: MeOH λ_max nm (logε): 281 (3.02), 274 (3.04), 213 (4.08); IR: KBr v_max cm⁻¹: 3480, 2950, 1730, 1600, 1580, 1470, 770; ¹H NMR (90 MHz, CDCl₃): δ 2.46–2.56 (2H, m, H-2), 2.65–2.77 (2H, m, H-3), 3.71 (3H, s, 1-COOCH₃), 7.45–7.92 (4H, m, H-3’–H-6’); EI-MS 70 eV, m/z (rel. int.): 236 [M]⁺ (0.4), 221 [M–CH₃]⁺ (0.4), 205 [M–OCH₃]⁺ (3.5), 149 [M–CH₂CH₂COOCH₃]⁺ (100).

**OSB diimidazolide** (10) (i.e. N-[4-[2’-(N-imidazolyl)carbonyl-phenyl]-4-oxobutyryl]imidazole).

OSB (2.5 mmol) and 1,1’-carbonyldimidazole (5.5 mmol) were dissolved in dry peroxide-free THF

<table>
<thead>
<tr>
<th>Compound</th>
<th>R_f value in chromatography system 1</th>
<th>R_f value in chromatography system 2</th>
<th>R_f value in chromatography system 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSB (1)</td>
<td>0.90</td>
<td>0.14</td>
<td>0.07</td>
</tr>
<tr>
<td>“Aliphatic” OSB methyl ester (2)</td>
<td>0.93</td>
<td>0.30</td>
<td>0.14</td>
</tr>
<tr>
<td>“Aromatic” OSB methyl ester (3)</td>
<td>0.93</td>
<td>0.26</td>
<td>0.24</td>
</tr>
<tr>
<td>OSB dimethyl ester (4)</td>
<td>0.95</td>
<td>0.75</td>
<td>0.38</td>
</tr>
<tr>
<td>“Aliphatic” OSB CoA (5)</td>
<td>0.47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>“Aromatic” OSB CoA (6)</td>
<td>0.47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OSB di-CoA (7)</td>
<td>0.34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CoASH</td>
<td>0.39</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>“Aromatic” OSB imidazolide (9)</td>
<td>0.69</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>OSB spirilactone (11)</td>
<td>0.92</td>
<td>0.63</td>
<td>0.29</td>
</tr>
<tr>
<td>DHNA</td>
<td>−</td>
<td>0.44</td>
<td>0.28</td>
</tr>
</tbody>
</table>

(Compounds 8 and 10 are not listed because they suffered hydrolysis in these systems).
(20 ml). N₂ was bubbled through the solution (5 h, 35 °C). The solvent was evaporated, the dry yellow residue was powdered in a mortar and subsequently washed twice with ice-cold water (10 ml) in order to hydrolyze unreacted 1,1'-carbonyldimidazole and to remove imidazole [17]. H₂O was removed by filtration, the residue was finally washed with light petroleum (60–80 °C, 5 ml) and dried in vacuo over P₂O₅; yield 1.93 mmol (77%) of 10, m.p. 159 °C.

Elemental analysis gave: C, 62.84; H, 4.46; O, 15.5; N, 17.1%; C₁₇H₁₆O₄N₄ requires: C, 63.35; H, 4.38; O, 14.89; N, 17.38%.

UV: THF λmax nm(logε): 281 (3.09), 272sh (3.19); IR: KBr νmax cm⁻¹: 3085, 2935, 2000, 1925, 1785, 1720, 1600, 1530, 745; ¹H NMR (300 MHz, DMSO-d₆): δ 2.07 (2H, s, H-2'), 2.92–3.16 (2H, m, H-3), 6.99 (1H, s, H-4''), 7.29 (1H, s, H-5''), 7.63 (1H, s, H-5''), 7.85 (1H, s, H-2''), 7.75–7.99 (4H, m, H-3'-H-6'), 8.34 (1H, s, H-2''); EI-MS 70 eV, m/z (rel. int.): 322 [M]+ (1.5), 255 [M-2imidazolyl]+ (40), 199 [255-C₂H₄CO]+ (3), 159 [M-2imidazolyl-H-CO]+ (55), 131 [159-CO]+ (13), 95 [carbonylimidazole]+ (9), 68 [imidazole]+ (100).

Alcoholysis of 10 in MeOH [18] (2 d, 20 °C) yielded the OSB dimethyl ester (4) which was identified by TLC.

When the reaction time was reduced to 30 min a mixture of 8, 9 and 10 resulted.

“Aromatic” OSB imidazolide (9) (i.e. N-[2'-(3-carboxypropionyl)benzoylimidazole). The reaction was carried out as described for 10. After 5 h, H₂O (500 μl) and HCOOH (300 μl) were added and the solution kept at 35 °C for 30 min. The solvent was evaporated (high vacuum), the brownish residue was redissolved in THF (1 ml) and stored 24 h at −20 °C. White crystals were isolated and washed as described for 10; yield 1.13 mmol (45%) of 9, m.p. 176–180 °C.

Elemental analysis gave: C, 61.74; H, 4.43; O, 23.6; N, 10.2%; C₁₄H₁₂O₂N₂ requires: C, 61.76; H, 4.41; O, 23.51; N, 10.29%.

UV THF λmax nm(logε): 281 (3.04), 273 (3.07), 235 (3.98); IR KBr νmax cm⁻¹: 3170, 3120, 2940, 2915, 2840, 2000, 1780, 1700, 1685, 1595, 1495, 755; ¹H NMR (300 MHz, DMSO-d₆): δ 1.92–2.16 (2H, m, H-2), 2.84–3.14 (2H, m, H-3), 6.99 (1H, s, H-4''), 7.30 (1H, s, H-5''), 7.85 (1H, s, H-2''), 7.77–8.01 (4H, m, H-3'–H-6'), 12.35 (1H, broad s, 1-COOH; disappeared on addition of D₂O); EI-MS 70 eV, m/z (rel. int.): 272 [M]+ (0.5), 205 [M-imidazolyl]+ (15), 159 [205-H₂O-CO]+ (100), 68 [imidazole]+ (90).

Alcoholysis of 9 in MeOH [18] yielded the “aromatic” OSB methyl ester (3), identified by TLC.

“Aromatic” OSB CoA ester (6) (i.e. 2'-(3-carboxypropionyl)benzoyl CoA):

CoASH (or dephospho CoASH, or deaminoo CoASH) (14 μmol) and 9 (25 μmol) were dissolved in THF (250 μl) and NaHCO₃ (400 μl, 0.1 M). The pH was adjusted to 8.0 with NaHCO₃ (1 mol) and N₂ was bubbled through the solution (5 h, 20 °C) [19], then HCOOH (100 μl) was added. The reaction mixture was separated either by PC (system I) or by semipreparative HPLC (column 250 × 8 mm; solvent A = H₂PO₄ (0.15 m) in H₂O, B = H₂PO₄ (0.15 m) in MeOH; gradient from 15 to 35% B in 25 min; 2.5 ml/min; tR(6) = 25.4 min; yield 9.3 μmol).

UV spectra were recorded directly in the eluent; λmax nm(logε): 257 (4.18).

“Aliphatic” OSB CoA ester (5) (i.e. 4-(2'-(carboxyphenyl)-4-oxobutyl) CoA) and OSB diCoA ester (7):

A mixture of 8, 9 and 10 (ca. 25 μmol) was reacted with CoASH (14 μmol) as described for 6. Separation of products was achieved by semipreparative HPLC (same conditions as for 6; tR(5) = 28.2 min, tR(6) = 25.4 min, tR(7) = 29.2 min). The concentrated CoA ester fractions were rechromatographed. Yields: 1500 nmol of 5, 170 nmol of 6, and 56 nmol of 7.

Yields are low because 8, 10 and the CoA esters formed undergo hydrolytic decomposition.

UVλmax nm(logε): 257 (4.18) for 5 and 258 (4.50) for 7. Starting with pure 10 did not yield pure 7 but a mixture of 6 and 7 due to partial hydrolysis of 10 at pH 8.0.

“Aromatic” [1-¹⁴C]OSB CoA (6):

[¹⁴C]OSB (1 μmol, 0.26 MBq) and CDI (6 μmol) were reacted in dry THF (1 ml, 2 d, 25°, N₂) yielding radioactive 10. For selective hydrolysis and simultaneous purification, the complete mixture was acidified with HCOOH (30 μl) and chromatographed in system 1 (Table I). Elution of the radioactive band at Rₜ 0.69 with H₂O yielded radiochemically pure “aromatic” [¹⁴C]OSB imidazolide (9), which was identified by methanalysis (2 d, 20°); only the “aromatic” [¹⁴C]OSB methyl ester (3) was formed.
The radioactive 9 (0.74 μmol, 0.192 MBq) was reacted with CoASH (8.6 μmol) as described for unlabelled 6; radiochemically pure 6 (0.34 μmol, 2.4 μCi) was isolated by analytical HPLC, \( t_R(6) = 20.0 \text{ min} \).

[1-\(^1\text{C}\)]labelled 5 and 7 could be obtained from a mixture of [1-\(^1\text{C}\)]labelled 8, 9 and 10 as described for unlabelled 5 and 7. Enzymic synthesis of radioactively labelled 5 and 7 proved to be more efficient (R. Kolkmann and E. Leistner, forthcoming publication).

**Alkaline hydrolysis of CoA esters**

The solution was adjusted to pH 11.5 with NaOH (2 m, 10 min, 30 °C) and subsequently readjusted to pH 1–2 with HCl (2 m).

**Determination of the molar ratio of OSB and CoA in CoA esters**

After alkaline hydrolysis of [1-\(^1\text{C}\)]OSB CoA esters, the solutions were examined by analytical HPLC; conditions are given under synthesis of 5 and 7. Separation was monitored at two wavelengths: 257 nm for CoASH and 237 nm for OSB. Compounds were identified by their retention time: \( t_R(\text{CoASH}) = 4.0 \text{ min} \), \( t_R(\text{OSB}) = 14.8 \text{ min} \). The amount of [1-\(^1\text{C}\)]OSB was determined in a liquid scintillation counter and calculated from the known specific activity of OSB employed in the synthesis of the ester; for CoASH, the peak area of the HPLC elution profile was compared with peak areas of known amounts of CoASH.

Molar extinction coefficients were calculated by UV spectroscopy using the known specific activity (0.26 MBq/μmol) of OSB.

**HPLC chromatography (Fig. 2, 3 and 4)**

In each case, separation was carried out on Lichrosorb RP-8 (7 μm, 250 × 4 mm). The following solvent systems were used:

Fig. 2: Solvent A: MeOH/H\(_2\)O (1:1) with 0.05 m tetrabutylammonium phosphate (TBAP).

Solvent B: H\(_2\)O with a gradient from 20 to 10%, pH 5.5. Flow: 1 ml/min, injection volume 20 μl.

Fig. 3: Solvent (isocratic): MeOH/H\(_2\)O 39:61 with 0.05 m tetrabutylammonium phosphate pH 6.9. Flow: 1 ml/min, injection volume 20 μl.

Fig. 4: Solvent A: H\(_3\)PO\(_4\) (0.15 m).

Solvent B: MeOH with H\(_3\)PO\(_4\) (0.15 m) with a gradient (...) from 12 to 25%. Flow: 1 ml/min, injection volume 160 μl.

**Results and Discussion**

Since OSB (1) has two carboxyl groups there are three possible structures for the naturally occurring OSB CoA ester: either the “aliphatic” (5), or the “aromatic” (6), or both carboxyl groups (7) are esterified with CoA.

Work with the enzymically formed coenzyme A ester showed that only one of the two carboxyl groups of OSB (1) is activated in the naturally occurring ester [4, 20]. In spite of this, it seemed to be desirable to prepare also the dicoenzyme A ester (7) of OSB to corroborate previous findings [4, 20].

It was also known from previous work [4, 20], that the enzymically formed coenzyme A ester is highly unstable (half-life: 15 min at 30 °C and pH 6.5 [4]). It was to be expected that the activated OSB derivative needed for synthesis of this CoA ester would be even more unstable and thus quite difficult to handle.

Different procedures are available for the synthesis of coenzyme A esters via activated acid derivatives. The most widely used energy-rich acid derivatives are acid chlorides [21], acid anhydrides [22], thiophenol esters [23] and N-hydroxy-succinimid esters [23, 24].

We failed, however, to prepare activated OSB (1) by these methods. Usually the spirolactone (11) of OSB was formed. Preparation of the N-acyl imidazoles of OSB, however, turned out to be method of choice.

First, OSB diimidazolide (10) was prepared and its structure confirmed by data given under Materials and Methods; especially MS (M\(^+\), m/z = 322) and \(^1\)H NMR signals clearly indicated the proposed structure. In addition, we prepared the OSB dimethyl ester (4) by methanolyis of 10 [18]. Staab [25] observed that aromatic imidazolides are more stable than aliphatic ones. Thus the OSB diimidazolide (10) was treated with THF/dil. H\(_2\)OAc, pH 3.2. Under these conditions of mild acid hydrolysis a selective cleavage of the imidazolide bond at the succinyl side chain of OSB took place, resulting in „aromatic” OSB imidazolide (9). Evidence for its proposed structure was obtained by MS (M\(^+\), m/z = 272), and by methanolyis of 9 which yielded selectively the
"aromatic" OSB methyl ester (3) which can be distinguished from the "aliphatic" OSB methyl ester (2) [11]. The 1H NMR signals of the imidazolylprotons of 9 corresponded to those of a synthetically prepared structural analogue of 9 viz. o-acetylbenzoyl imidazol and are different from an "aliphatic" imidazol viz. N-acetyl imidazol [26].

Because of its greater instability relative to 10, the "aliphatic" OSB imidazolide (8) could not be isolated as a pure single substance, but a mixture of all three imidazolides 8, 9 and 10, consisting predominantly of the "aliphatic" derivative (8), was obtained by reducing the reaction time of OSB and CDI to about 30 min.

Therefore the mixture of imidazolides was converted to a mixture of its coenzyme A esters and the esters were separated by HPLC. Different techniques have been described for separating CoA esters and CoASH from each other or from impurities. LC on Sephadex G 10 [27], polyamide [28, 29], or DEAE-cellulose [30] is very time-consuming and effected only poor resolution.

In our hands, PC [3, 13] and TLC [11] proved satisfactory to free OSB CoA esters from other reaction components, including free acid and contaminants from incubation mixtures. PC could even be used for semi-preparative separations (up to 10 mg CoA ester/sheet). In order to obtain both short analysis time and good resolution we used two HPLC systems. HPLC of CoASH and similar compounds on reversed phase material (RP-8) with simple MeOH–H2O mixtures results in broad elution profiles, unresolved substance peaks and considerable tailing [31], since CoA has low affinity to the alkyl-bonded packing. However, there are two general methods to improve separation of these compounds. In RP-ipc, addition of the ion-pair reagent TBAP increases the retention of CoA derivatives due to interaction of the counterion TBA+ with negatively charged groups in the CoASH molecule, thus forming a more lipophilic, uncharged complex [32, 33]. This results in baseline separation of all compounds of interest as shown in Fig. 2. Even very similar compounds could be sharply separated, e.g. the "aromatic" and the "aliphatic" CoA ester of OSB.

Fig. 3 shows the isocratic separation of a mixture of structurally related compounds, viz. the "aromatic" OSB CoA ester (6), the "aromatic" OSB dephospho-CoA ester and the "aromatic" OSB deamino-CoA ester.

This technique results in satisfactory resolution of CoA derivatives, but it is restricted to analytical work, because the ion-pair reagent (TBAP) impedes further examination of the separated CoA esters (e.g. enzyme studies, UV spectra, FAB mass spectra).

The removal of TBAP is rather time-consuming [31] and would result in decomposition of the unstable CoA esters. Moreover, a nearly neutral pH is required for RP-ipc, at which the CoA esters, especially the "aliphatic" OSB CoA (5) and the OSB di-CoA (7) are quite unstable [vide infra, 20]. At lower pH (1 to 2), the stability of the CoA esters improves considerably, but the effect of TBA+ decreases since there is no possibility to interact with negatively charged groups in the coenzyme A moiety of the ester.

For preparative isolation and also for routine analysis, we used a different method: Increase of the ionic strength of the eluent by addition of phosphate ions was also reported to improve separation on re-
Fig. 3. Separation of analogues of OSB coenzyme A ester (6) by reversed-phase ion-pair chromatography. (a) OSB, 2 nmol; (b) dephospho-CoASH, 0.5 nmol; (c) deaminocoASH, 1 nmol; (d) CoASH, 0.6 nmol; (e) “aromatic” OSB dephospho-CoA ester, 1.15 nmol; (f) “aromatic” OSB deaminocoA coenzyme A ester, 1.5 nmol; (g) “aromatic” OSB CoA ester (6), 1.5 nmol.

versed phase material [29, 34]. We found that 0.15 M H₃PO₄ (pH 1.5) in the eluent was ideal both for baseline separation of CoA derivatives and other components (Fig. 4) and for stability of the OSB CoA esters. Replacement of H₃PO₄ by either HCOOH or HOAc resulted in major tailing and unresolved peaks. UV spectra could be recorded directly, and enzymic conversion (S. Kolkmann and E. Leistner, forthcoming publication) to DHNA was studied after removal of most of the MeOH in high vacuum and subsequent adjustment of the pH to 8.0 with NaOH.

For characterization as outlined below, the “aromatic” coenzyme A ester (6) was prepared from the “aromatic” imidazolide (9), the di-coenzyme A ester (7) from the di-imidazolide (10) and the “aliphatic” coenzyme A ester (5) from the mixture of imidazolides and subsequent separation of CoA esters by HPLC.

The three OSB CoA esters (5, 6, 7) were characterized by several methods: After paper chromatography the esters gave the delayed colour reaction typical of coenzyme A esters [13] when sprayed with nitroprusside reagent and subsequently with base. The release of coenzyme A from the ester was also monitored photometrically at 412 nm using Ellman’s reagent [35, 36]. The esters were kept at 30 °C and pH 7.5 (Fig. 5). The “aliphatic” ester (5) turned out to be highly unstable (half-life 7.5 min). The “aromatic” ester (6), however, was much more stable and had a half-life of 8.5 h. The enzymically formed OSB CoA ester [4] previously described also was highly unstable under similar conditions. This is a strong indication that the naturally occurring and enzymically formed [4, 20] OSB CoA ester is identical with the “aliphatic” coenzyme A ester (5).

When the synthetic coenzyme A esters (5, 6 and 7) were hydrolyzed under alkaline conditions, compounds were formed which cochromatographed (HPLC) with OSB (1) and CoASH. The molar ratio OSB:CoASH was 1:0.97 for 5, 1:0.96 for 6 and

Fig. 4. HPLC analysis of potential substrates and coenzymes of vitamin K₂ biosynthesis with H₃PO₄ in the mobile phase. (a) ATP, 1.2 nmol; (b) CoASH, 1.2 nmol; (c) OSB, 10 nmol; (d) OSB spirrolactone (11), 20 nmol; (e) “aromatic” OSB CoA ester (6), 1.8 nmol; (f) “aliphatic” OSB CoA ester (5), 1.8 nmol; (g) OSB dicoenzyme A ester (7), 0.8 nmol.
Fig. 5. Photometrically recorded formation of the mercaptidion formed from 5,5′-dithiobis-(2-nitrobenzoic acid) (i.e. Ellman's reagent) and coenzyme A released from the "aromatic" (6) or "aliphatic" (5) coenzyme A ester of OSB (1) at pH 7.5 and 30 °C. Although the latter value indicated that some of the CoASH formed was oxidized to the bis-coenzyme A, it is reasonable to assume that 7 consists of OSB (1) and CoASH in a 1 to 2 molar ratio. This is also indicated by the UV-spectra: The molar extinction coefficient $\varepsilon_{235}$ of 7 (32000) is twice that of 5 (15090) and 6 (15100) (Fig. 6) and is congruent with that of a 1:2 mixture (31000) of OSB and CoASH. The UV-spectra of 5 and 6 (Fig. 6) resemble those of a 1:1 mixture of OSB and CoASH. The "aliphatic" coenzyme A ester (5) was distinguished from the "aromatic" coenzyme A ester (6) after a synthesis which gave 6 alone (see above). Moreover, there is a characteristic contribution of the thioester bond to the UV absorbance of coenzyme A esters. The UV absorption is maximal at about 235 nm for "aliphatic" esters [30, 37] and at about 269–300 nm for "aromatic" esters [23, 38]. The difference spectra (i.e. absorbance before minus absorbance after esterolysis) confirmed that 5 is indeed the "aliphatic" OSB coenzyme A ester ($\Delta\lambda_{\text{max}} = 235$ nm; $\Delta\varepsilon_{235} = 3670$ [mol$^{-1}$cm$^{-1}$]) (Fig. 7). The structurally related succinyl coenzyme A ester shows $\Delta\varepsilon_{235} = 4000$ [mol$^{-1}$cm$^{-1}$] [30].

For the "aromatic" coenzyme A ester (6), the loss of absorption due to thioester disruption caused a difference spectrum with a maximal absorption $\Delta\varepsilon_{291} = 550$ [mol$^{-1}$cm$^{-1}$] (Fig. 8), indicating the presence of an "aromatic" thioester bond. Molecular weight and fragmentation of the OSB coenzyme A
Fig. 7. Difference spectrum of "aliphatic" OSB CoA ester (5). (A) UV spectrum before (-----), after (----) alkaline hydrolysis of the CoA ester (5) (pH 11.5, 30 °C, 10 min). (B) Difference spectrum; recorded with equal concentrations of intact (5) in sample cell against hydrolyzed (5) in reference cell; $\Delta\lambda_{\text{max}} = 235$ nm.

Fig. 8. Difference spectrum of "aromatic" OSB CoA (6). (A) UV spectrum before and after (——) alkaline hydrolysis (pH 11.5, 30 °C, 10 min). There is hardly any difference because of the rather low $\Delta$E291 = 550 [mol·l·cm$^{-1}$]. (B) Difference spectrum; recorded with equal concentrations of intact (6) in sample cell against hydrolyzed (6) in reference cell, indicating $\Delta\lambda_{\text{max}} = 291$ nm.
Fig. 9. FAB⁻ mass spectrum (negative ions) of “aliphatic” OSB CoA ester (5) (A) and FAB⁺ mass spectrum (positive ions) of “aliphatic” OSB CoA ester (5) (B).
esters were also determined by fast atom bombardment (FAB) mass spectrometry [39]. The calculated molecular weight of both mono coenzyme A esters is $M_r = 971.72$. In both cases molecular and pseudomolecular ions and fragments were observed which are in agreement with the proposed structure. Since the “aliphatic” coenzyme A ester (5) is the more labile one, only the negative (Fig. 9A) and positive (Fig. 9B) ions of this compound are shown.

The base peak (Fig. 9A) is the pseudomolecular ion $[M-H]^+$. Further ions in the pseudomolecular region are caused by attachment of $\text{Na}^+$ and $\text{K}^+$. The ion with $m/z$ 766 is due to loss of the $o$-succinylbenzoyl residue, $m/z$ 426 is 3'-phospho-AMP and $m/z$ 506 corresponds to 3'-phospho-ADP. Both fragments are generated from the coenzyme A-residue of 5. Fragment $m/z$ 560 is ascribed to cleavage of the pyrophosphate bond in the coenzyme A part.

Fig. 9B shows the positive ions with $m/z$ 972 representing the pseudomolecular ion $[M+H]^+$. FAB mass spectra of the “aromatic” coenzyme A ester (6) are similar since cleavage of bonds occurred, as expected, at heteroatoms only. It will be shown in a forthcoming publication that, contrary to previous work [6, 11], the “aliphatic” coenzyme A ester (5) alone is the intermediate in vitamin K$_2$ biosynthesis. The “aromatic” (6) or the di-coenzyme A ester (7) of OSB (1) are not involved in vitamin K$_2$ biosynthesis [40].

Acknowledgements

The financial support of the Deutsche Forschungsgemeinschaft, the Minister für Wissenschaftliche Forschung des Landes Nordrhein-Westfalen and Fonds der Chemischen Industrie is gratefully acknowledged. We are grateful to Drs. H. Egge and J. Peter-Katalinić, Institut für Physiologische Chemie, Nußallee 11, 53 Bonn 1, FRG, for the FAB mass spectra and to Dr. H. Egge for his help in the preparation of the manuscript.