Enzymatic Synthesis, Characterization, and Metabolism of the Coenzyme A Ester of o-Succinylbenzoic Acid, An Intermediate in Menaquinone (Vitamin K₃) Biosynthesis*

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The enzymatic synthesis of the "active" o-succinylbenzoic acid is described and the factors controlling its formation are investigated. Tritium-labeled coenzyme A is incorporated into "active" o-succinylbenzoic acid, but label from [2-3H]ATP or [γ-32P]ATP is not, indicating that the active compound is a coenzyme A thio ester (2).

The compound is shown by two different methods to contain 1 mol only of coenzyme A per mol of o-succinylbenzoic acid. The o-succinylbenzoic acid coenzyme A ester (2) is unstable at alkaline and neutral pH, but is fairly stable under acid conditions. The coenzyme A ester (2) is converted to 1,4-dihydroxy-2-naphthoic acid (3) by enzyme preparations from Mycobacterium phlei and Escherichia coli without any cofactor requirement.

It is generally accepted that o-succinylbenzoic acid (1) (4-(2'-carboxyphenyl)-4-oxobutanoic acid) is an intermediate in the biosynthesis of menaquinones (4) (Fig. 1) and of a series of plant naphtho- and anthraquinones (1–3). The cell-free synthesis of OSB (1) has been demonstrated (4) and its enzymic conversion to 1,4-dihydroxy-2-naphthoic acid (3) has been shown to be catalyzed by enzyme extracts from bacteria (5, 6). This reaction, which requires ATP, coenzyme A, and Mg²⁺, is catalyzed by two different enzymes, namely o-succinylbenzoate:coenzyme A ligase and naphthoate synthase (6).

The product of the former enzyme is a mono-coenzyme A thio ester (2) (7) (OSB CoA-ester) whose aromatic carboxyl group is activated (8). Compound (2) is the substrate of the second enzyme.

Investigations on the intermediacy of the postulated activated derivative of OSB have now resulted in the isolation of this highly unstable compound. A preliminary account of part of this work has been published (7). Further experimental details on the nature of "active" OSB, its enzymic synthesis, and metabolism are now presented.

MATERIALS AND METHODS

Bacteria—The strains Escherichia coli K12 and Mycobacterium phlei were obtained from the Institut für Mikrobiologie of this University.

Enzymes and Chemicals—o-Succinyl[1-14C]benzoic acid was synthesized as described by Dananet (9). [G-3H]Coenzyme A was purchased from New England Nuclear, [2-3H]ATP and [γ-32P]ATP from Amersham-Buchler, Braunschweig (F.R.G.). Enzymes (phosphotransacetylase, EC 2.3.1.8; citrate synthase, EC 4.1.3.7; malate dehydrogenase, EC 1.1.1.37; deoxyxynucleosidas 1, EC 3.1.21.1) were obtained from Sigma. The synthesis of DHNA (5) and its derivatives has been described (5).

Growth of Organism—E. coli was maintained on agar slants (Standard-I-Nähragar, Merck, Darmstadt, F.R.G.) for 24 h at 37 °C and then used to inoculate a preculture (50 ml) in an Erlenmeyer flask (300 ml). Incubation was carried out for 24 h at 37 °C on a gyratory shaker (150 rpm). A sample (2.5 ml) of the bacterial suspension was withdrawn and used to inoculate the main culture which was maintained under the same conditions. The bacteria were harvested after 3 h of incubation and centrifuged (5000 × g) for 10 min. The pellet was resuspended in potassium buffer (0.05 M, pH 6.9) and centrifugation was repeated. The supernatant was discarded and the bacteria were stored at −20 °C. The media of both the preculture and the main culture had the same composition: meat extract (1.5 g/liter), yeast extract (1.5 g/liter), peptone from casein (5.0 g/liter), NaCl (2.5 g/liter), KH₂PO₄ (3.5 g/liter), K₂HPO₄ (1.2 g/liter), glucose (4.0 g/liter), pH 7.0–7.2.

M. phlei was kept on agar slants (Standard-I-Nähragar, Merck, Darmstadt, F.R.G.) for 48 h at 37 °C and then used to directly inoculate the culture medium (10 ml) without preculture. The cultures were incubated for 48 h at 37 °C on a gyratory shaker at 150 rpm. The cells were harvested by centrifugation (10 min at 5000 × g) and washed three times with water. The bacteria were stored at −30 °C.

Preparation of an Enzyme Extract from M. phlei Catalyzing the Conversion of OSB (1) into OSB CoA-ester (2) and of ODS CoA-ester (3) into DHNA (5)—Frozen cells (10 g) were thawed and suspended in 3-(N-morpholino)propanesulfonic acid buffer (15 ml, 0.02 M, pH 6.9) containing dithiothreitol (0.2 mM) and dimethyl sulfoxide (20%). The cell suspension was cooled in an ice salt mixture and sonicated (Branson Sonifier, Branson Sonic Power Co., Danbury, CN) 10 times for 60 s with 60 watts. Thirty-s intervals were allowed to elapse between repeated sonications. The supernatant of centrifugation at 50,000 × g (10 min) gave a solution containing 5.0 mg of protein per ml. Protein was determined by a published method (11). For conversion of (2) to (3), this protein extract was then centrifuged through Sephadex (7) and used directly.

For conversion of (2) to (3), however, the protein solution (10 ml) was cooled (0 °C) and stirred, and a potassium sulfate solution (6 ml, 2% in 3-(N-morpholino)propanesulfonic acid buffer, pH 6.9, 37 °C) containing dimethyl sulfoxide (20%) and diethiothreitol (0.2 mM) was added with stirring over 10 min at 0 °C (12).

The enzyme solution was stirred (20 min) and centrifuged (10 min, 12,000 × g) and then passed through Sephadex G-25 (12). Both preparations remained active at −20 °C for not less than 6 weeks.

Preparation of an Enzyme Extract from E. coli Catalyzing the Conversion of OSB CoA-ester (2) into DHNA (5)—Frozen cells (7 g) were thawed and suspended in potassium phosphate buffer (20 ml, 0.05 M, pH 6.9) containing EDTA (10 mM) and diethiothreitol (0.2 mM). The cells were cooled (0 °C) and sonicated 10 times for 10 s at 60 watts with 50-s intervals between sonications. After addition of deoxyxynucleosidas (0.8 mg), MgCl₂ in H₂O (1 M, 0.4 ml), and [N2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine-NaOH (1.4 ml, 1 M, pH 8.5), centrifugation was carried out for 10 min at 30,000 × g. The supernatant contained 20 mg of protein in 1 ml. The enzyme
preparation remained active at -20 °C for not less than 6 weeks.

Isolation of the OSB CoA-ester (2)—The incubation of OSB with the enzyme extract from M. phlei was terminated by addition of formic acid (100 μl) and concentrated at 10⁻¹ torr and room temperature. The residue was either applied to Whatman No. 3MM chromatography paper (washed with water) or to cellulose thin layer plates (Merck, Darmstadt, F.R.G.). The chromatograms were developed in 1-butanolacetic acidwater (5:2:3). The OSB CoA-ester, RF 0.5, was detected by radioactivity scanning (7) and eluted from formic acid (3 μl). When [G⁻¹H]coenzyme A in addition to o-succinyl[¹⁴C]benzoic acid was used, the eluate was evaporated to dryness, and the residue was dissolved in formic acid (0.1 mM) containing EDTA (1.0 mM) and applied to a mercury-Sepharose column (0.55 × 6 cm, Affi-Gel 501, Bio-Rad). Residual coenzyme A was retained on the column, whereas the OSB CoA-ester was eluted (0.1 μl formic acid, 1 mM EDTA) with the void volume. For final purification, the paper or thin layer chromatographic step was repeated.

Investigations on the Stability of OSB CoA-ester (2)—The isolated [¹⁴C]OSB CoA-ester was dissolved either in formic acid (8 μl, pH 1.6) or in potassium phosphate buffer (0.1 μl, pH 6.5) and the solution was then kept at either 0 °C or 30 °C. Samples were withdrawn from these solutions at intervals (Fig. 3) and were chromatographed after addition of carrier OSB (1) and OSB spirodilactone (5). Chromatography was carried out on cellulose plates using 1-butanolacetic acidwater (5:2:3) as the solvent system. Decomposition of the ester was recorded after elution of intact ester, OSB, and its spirodilactone and scintillation counting of the eluates. At neutral pH, only OSB and a compound co-chromatographing with coenzyme A were observed. At neutral pH, the spirodilactone and only traces of OSB were detected.

Identification of DHNA (3) Synthesized Enzymically from OSB CoA-ester (2)—A methanolic solution containing 1,4-dihydroxy-2-naphthoic acid (3) was added as carrier to the acidified (6 N HCL) incubation mixture, and the DHNA (3) was extracted into ether. Chromatography on silica gel in chloroform:methyl acetate:formic acid (45:6.5:6.0:5) and radioactivity scanning revealed a radioactive peak coinciding with authentic 1,4-dihydroxy-2-naphthoic acid (3) (Fig. 4). The radioactive 1,4-dihydroxy-2-naphthoic acid was eluted (MeOH) and methylated with diazomethane (1 min, 0 °C). Chromatography on silica gel in toluene:acetic acid (9:1) revealed a radioactive peak coinciding with an authentic sample of the methyl ester of (3). The methyl ester of (3), as well as 1-hydroxy-4-methoxy-2-naphthoic acid methyl ester which was obtained from the former compound by prolonged treatment with diazomethane, was identified by radioisotope paper chromatography (Varian AEC Chromatotron 1400), equipped with a column (6 feet × 0.125 inch outside diameter, stainless steel) of 10% SE 30.

Helium (30 ml/min) was passed through the column. The injector and detector were kept at 200 °C and 275 °C, respectively. The column was kept at 200 °C. The effluent of the column was passed through a stream-splitter which channelled 10% of the effluent into a flame ionization detector and 90% through a heated tube into a radioactivity counting system (Model RGC 170, Perkin-Elmer).

RESULTS

Enzymic Synthesis of the Coenzyme A Ester of o-Succinylbenzoic Acid (2)—It has previously been shown that enzyme extracts of E. coli and M. phlei catalyze the ATP-, CoASH-, and Mg⁺⁺-dependent conversion of OSB (1) to 1,4-dihydroxy-2-naphthoic acid (3) (5, 6). This conversion is catalyzed by two different enzymes (6), o-succinylbenzoate:coenzyme A ligase and naphthoate synthase. When the latter enzyme is removed from a crude enzyme extract with the aid of protamine sulfate and the supernatant incubated (Table I) in the presence of ATP, CoASH, Mg⁺⁺, and o-succinyl[¹⁴C]benzoic acid (1), formation of the activated derivate (2) of o-succinylbenzoic acid can be observed (Table I, Figs. 1 and 2). As will be shown later, this activated compound is very likely the coenzyme A ester of o-succinylbenzoic acid (2). Under the conditions used, the yield of (2) ranges from 20 to 30% with reference to the OSB (1) employed. Formation of the OSB CoA-ester (2) depends on the presence of ATP, CoASH, Mg⁺⁺, OSB (1), and enzyme, which is not inactivated by heat (Table I).

We had to take into account that either of the two or both carboxyl groups of o-succinylbenzoic acid would be activated.

In principle, a carboxyl group may be activated as a thio

| Table 1 |
|---|---|---|
| Incubation mixture | OSB CoA-ester (2) | Relative activity |
| --- | --- | --- |
| Complete | 940 | 100 |
| -Mg⁺⁺ | 195 | 20.8 |
| -ATP | 12 | 1.3 |
| -CoASH | <4 | <0.4 |
| Complete, heat-inactivated enzyme | <4 | <0.4 |

Fig. 2. Time-dependent formation of OSB CoA-ester (2) and OSB spirodilactone (5) from OSB (1). Disappearance of OSB (1) from the incubation mixture and sequential appearance of OSB CoA-ester (2) and OSB spirodilactone (5). The incubation mixture contained in a final volume of 245 μl of 1,4-dihydroxy-2-naphthoic acid (13.8 nmol = 0.1 μCi), ATP (2 μmol), CoASH (0.1 μmol), MgCl₂ (4 μmol), and potassium phosphate (5.0 μmol, pH 6.5) in a final volume of 70 μl. Incubation was carried out for 10 min at 30 °C.
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ester or as an anhydride. In the former case, the OSB might be found to the —SH group of an enzyme or to the —SH group of coenzyme A. In the latter case, an orthophosphoric acid anhydride or an AMP or ADP derivative may be formed. The activated intermediate of OSB is not an enzyme-bound derivative because its chromatographic behavior (13) strongly suggests that it is a coenzyme A ester. Experiments listed in Table II exclude all but one of the remaining possibilities. When the o-succinylbenzoate:coenzyme A ligase is incubated in the presence of [γ-3P]- or [2-3H]ATP, no label in the activated intermediate (2) is observed. Label from [G-3H]coenzyme A, however, is clearly detected (Table II). This finding was confirmed by another experiment in which the [14C]OSB-labeled coenzyme A ester (2) was hydrolyzed under alkaline conditions and the OSB, as well as the coenzyme A, was determined quantitatively. To determine the amount of OSB, the radioactivity in the hydrolysate was measured and the amount of OSB present was then calculated from the known specific activity of the o-succinylbenzoic acid employed in the enzymic synthesis of the OSB CoA-ester. The amount of coenzyme A in the hydrolysate was determined enzymatically by a reaction in which phosphotransacetylase (EC 2.3.1.8), citrate synthase (EC 4.1.3.7), and malate dehydrogenase (EC 1.1.1.37) were used in a coupled assay (14). The system was calibrated using known amounts of coenzyme A. The result showed that o-succinylbenzoic acid and coenzyme A were present in the hydrolysate in a molar ratio of 1:1.02. This experiment also shows unequivocally that the intact coenzyme A molecule is the activating residue in the o-succinylbenzoic acid coenzyme A ester (2) because the phosphotransacetylase which converts acetyl phosphate and coenzyme A to acetyl coenzyme A and inorganic phosphate is specific for coenzyme A (14).

Stability of the OSB CoA-ester—When incubations were carried out under conditions outlined in Table I, formation of the spirodilactone (5) of o-succinyl benzoic acid was also observed. This is consistent with previous findings (5, 6). The formation of (2) and (5) with time is shown in Fig. 2. As can be seen, o-succinylbenzoic acid gradually disappears from the incubation mixture while the OSB CoA-ester (2) and spirodilactone (5) are rapidly formed. The sequential appearance of the maxima of compounds (2) and (5) is consistent with the assumption (5, 5) that (5) is a product of (2). Decomposition of the isolated OSB CoA-ester (2) to OSB spirodilactone (5) was also observed (7). This decomposition is temperature- and pH-dependent (Fig. 3). At alkaline pH, the ester was found to

![Fig. 3. Stability of the OSB CoA-ester (2). Stability of the OSB CoA-ester (2) as influenced by pH and temperature. Experimental details are given under “Materials and Methods.”](http://www.jbc.org/)

### Table II

**Attempts to incorporate label from [G-3H]coenzyme A, [2-3H]ATP, or [γ-3P]ATP into the “active” OSB derivative**

The incubation mixture contained in a final volume of 70 μl: [1-14C]OSB, ATP (0.5 pmol unless otherwise given), CoASH (0.125 pmol unless otherwise given), MgCl2 (1 μmol), protein (75 μg). Incubation was carried for 20 min at 30°C and pH 6.5. Further experimental details are given in Table I and under “Materials and Methods.”

|                      | Amount   | Specific activity | 1H/14C ratio | Found  |
|----------------------|----------|-------------------|--------------|--------|
|                      | μmol     | pCi/μmol          |              |        |
| [1-14C]OSB           | 0.01     | 7.22              | 4.16         | 4.76   |
| [G-3H]Coenzyme A     | 0.025    | 30                |              |        |

| B. Nonincorporation of [2-3H]ATP |
|----------------------------------|
| Amount   | Specific activity | 1H/14C ratio | Found  |
|----------|-------------------|--------------|--------|
| [1-14C]OSB | 0.01     | 7.22         | 4.16  | 0.041 |
| [2-3H]ATP  | 0.05     | 30           |        |       |

| C. Nonincorporation of [γ-3P]ATP |
|----------------------------------|
| Amount   | Specific activity | 1H/14C ratio | Found  |
|----------|-------------------|--------------|--------|
| [1-14C]OSB | 0.01     | 7.22         | 7.22  | <333  |
| [γ-3P]ATP  | 0.05     | 1.00         |        |       |
The stability of the ester at pH 6.5, the half-life of the compound was 15 min at 30 °C yielding the OSB spirodilactone (5) as the product of decomposition. The stability of the ester at pH 6.5 increased at 0 °C (half-life > 2.0 h). At pH 1.6 and 0 °C, the stability was even greater. Under these conditions, the OSB CoA-ester can be stored for several days with only slight decomposition (Fig. 3). In spite of the observed instability of the OSB CoA-ester (2), it can be isolated by paper and thin layer chromatography (see Ref. 7 and "Materials and Methods").

The Role of the OSB CoA-ester (2) in Vitamin K$_2$ Biosynthesis—The experiments described above do not in themselves prove that compound (2) is involved in menaquinone (Vitamin K) biosynthesis. Conversion of (2) to 1,4-dihydroxy-2-naphthoic acid (3), however, would show that the OSB CoA-ester is indeed an intermediate in Vitamin K$_2$ biosynthesis.

![Conversion of OSB CoA-ester (2) to DHNA (3).](image)

**Fig. 4. Conversion of OSB CoA-ester (2) to DHNA (3).** Result of the incubation of the isolated [1-14C]OSB CoA-ester (2) with an enzyme extract from M. phlei. Formation of DHNA (3), OSB (1), and OSB spirodilactone (5), OSB CoA-ester (24,000 dpm) was incubated for 30 min at 30 °C and pH 8.0 with an enzyme preparation (1 mg of protein, final volume 0.2 ml) from M. phlei. The product DHNA (3) was identified as described under "Materials and Methods" and as shown in Fig. 5.

**Table III**

Conversion of the OSB CoA-ester (2) to DHNA (3) by an enzyme extract from M. phlei

| Incubation mixture | Formation of DHNA (3) pmol | % |
|--------------------|---------------------------|---|
| A. 1. OSB CoA-ester + enzyme | 202 | 100 |
| 2. OSB CoA-ester + enzyme + Mg$^{2+}$ | 185 | 92 |
| 3. OSB CoA-ester + enzyme + Mg$^{2+}$ + ATP | 206 | 102 |
| 4. OSB CoA-ester + enzyme + Mg$^{2+}$ + CoASH | 164 | 81 |
| 5. OSB CoA-ester + heat-inactivated enzyme | <2 | <1 |
| B. 1. OSB + enzyme + Mg$^{2+}$ + CoASH | <2 | <1 |
| 2. OSB spirodilactone + enzyme + Mg$^{2+}$ + CoASH | <2 | <1 |
| C. 1. OSB CoA-ester + enzyme + Mg$^{2+}$ + diamide | 24 | 12 |
| 2. OSB + enzyme + Mg$^{2+}$ + ATP + CoASH + diamide | 18 | 9 |

1,4-Dihydroxy-2-naphthoic acid (3) is the substrate for the prenylation reaction leading to demethylmenaquinone (15). The latter compound is the immediate precursor of menaquinone.

In Table III, results of experiments are listed which were designed to investigate the conversion of the OSB CoA-ester (2) to DHNA (3). An enzyme preparation from M. phlei readily converts the ester (2) to 1,4-dihydroxy-2-naphthoic acid (3) in 57% yield (Fig. 4 and Table III). When an enzyme preparation from E. coli was used, the yield was as high as 80% with reference to the ester (2) employed. The conversion to (3) does not require any cofactor. When Mg$^{2+}$ alone, Mg$^{2+}$ and ATP, or Mg$^{2+}$ and coenzyme A were added to the enzyme solution, the yield did not increase. No reaction took place when heat-inactivated protein was used (Table IIIA). It is possible, in principle, that the o-succinylbenzoic acid coenzyme A ester (2) might first hydrolyze and then be converted to DHNA (3). Experiments listed in Table III, B and C exclude this possibility. When an enzyme preparation catalyzing the conversion of OSB (1) to DHNA (3) is incubated with the same molar amounts of O-succinylbenzoic acid and coen-
zyme A which are also present in the activated intermediate (2), the yield of DHNA (3) was less than 2 pmol (Table III, Experiment B1). The intermediacy of the spirodilactone (5) is also excluded since no conversion to DHNA (3) was observed in the presence of spirodilactone, together with Mg2+ and coenzyme A. When Mg2+ and coenzyme A are added to a preparation containing o-succinyl-[1,14C]benzoic acid (1), conversion is observed only when ATP is also added (Table IIIIB). This conversion, however, is much less when compared to the standard assay mixture (Table IIIA) in which the o-succinylbenzoic acid coenzyme A ester (2) is employed as the substrate.

These data show that the conversion of (2) to (3) proceeds directly and without cofactors. Further support for this conclusion is presented in Table IIIIB, based on experiments in which azodicarboxylic acid bis-(dimethylamido) (‘‘diamide’’) was included in the incubation mixture. ‘‘Diamide’’ oxidizes a sulfhydryl group such as that present in coenzyme A to a disulfide (16). Since conversion of o-succinylbenzoic acid coenzyme A ester (2) into DHNA (3) takes place in the presence of ‘‘diamide,’’ free coenzyme A cannot be involved in the conversion. A control experiment (Table IIIIC) shows that OSB (1) is not converted into DHNA (3) when the coenzyme A present in the incubation mixture has been oxidized by means of ‘‘diamide.’’

The 1,4-dihydroxy-2-naphthoic acid (3) which was enzymically formed from the OSB coenzyme A ester (2) was identified in the following way. A radiochromatogram of the ethereal extract of an incubation mixture in which OSB CoA-ester (2) had been converted to 1,4-dihydroxy-2-naphthoic acid (3) is shown in Fig. 4. Of the three major peaks which appear, the main peak coincides with DHNA (3), another one with OSB spirodilactone (5), and a third one with OSB (1). Whereas DHNA is formed enzymically, the spirodilactone (5) is the product of the chemical degradation of the OSB CoA-ester (2) (see above and Ref. 7). The DHNA (3) was further identified as described under ‘‘Materials and Methods’’ and as shown in Fig. 5.

DISCUSSION

The experiments described here show that o-succinylbenzoic acid coenzyme A ester (2) can be isolated and characterized, notwithstanding previously encountered difficulties (5, 6). This makes it now possible to study in more detail the properties of two enzymes o-succiny1benzoate:coenzyme A ligase and naphthoate synthase. The coenzyme A ester (2) is formed with the participation of ATP and CoASH, but its further conversion to 1,4-dihydroxy-2-naphthoic acid (3) occurs without any cofactor requirements. The OSB CoA-ester (2) is a mono-coenzyme A derivative. The compound is very unstable at neutral pH. We assume that in the decomposition of the OSB CoA-ester (2) the carboxylate anion at C-atom 1 (see numbering in (1)) acts as a nucleophile which in the first instance attacks the keto group (C-atom 4) resulting in the formation of a lactone ring. A second lactone ring is then formed by attack of the carbonyl oxygen at the thio ester followed by elimination of the coenzyme A residue. If this mechanism is correct, decomposition of the OSB coenzyme A ester (2) should be substantially reduced at low pH when ionization of the carboxyl group would be minimized. The experiments presented in Fig. 3 demonstrate that this suggestion is very likely to be correct.

It has been assumed (5) that the aromatic rather than the aliphatic carboxyl group is activated in OSB (1). This assumption is correct (8). When the isolated OSB CoA-ester (2) was methylated (diazoethane) and the resulting o-methylthio ester was hydrolyzed under conditions which would preferentially cleave the thio ester group, OSB (1) was obtained with the aliphatic rather than the aromatic carboxyl group being methylated. This shows that coenzyme A is bound to OSB (1) via its aromatic carboxyl group. One has to keep in mind, however, that activation at the aliphatic carboxyl group is also very likely to occur in nature because Leete and Bodem (17) have shown that alkaloids occurring in orchids are derived from o-succinylbenzoic acid. The biosynthesis of these alkaloids is likely to proceed via reduction of the aliphatic carboxyl group. It is very well known that reduction of a carboxyl group may require previous activation with ATP and coenzyme A (18).

In the context of the present reaction sequence, a ring closure reaction with activation at the aromatic carboxyl group occurs. In agreement with a previous suggestion (19), we assume that the OSB CoA-ester enolizes prior to ring closure. This would result in a conjugated double bond system which would stabilize a carbanion at C-atom 2 (2). Nucleophilic attack of this carbanion at the ester group would result in elimination of coenzyme A and aromatization. The driving force of this ring closure reaction would probably also be supplied by the aromatization process. It has been proposed that the ring closure takes place in a manner analogous to a Dieckmann reaction (20). However, a Dieckmann type reaction requires activation at two carboxyl groups whereas in this case we have shown that only one carboxyl group is activated. We therefore believe that this ring closure reaction is hitherto unparalleled.

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