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Chemistry

# Biosynthesis of vitamin $B_{12}$: Factor IV, a new intermediate in the anaerobic pathway

**(tetradehydrocorrin/biosynthetic intermediate/NMR spectroscopy)**

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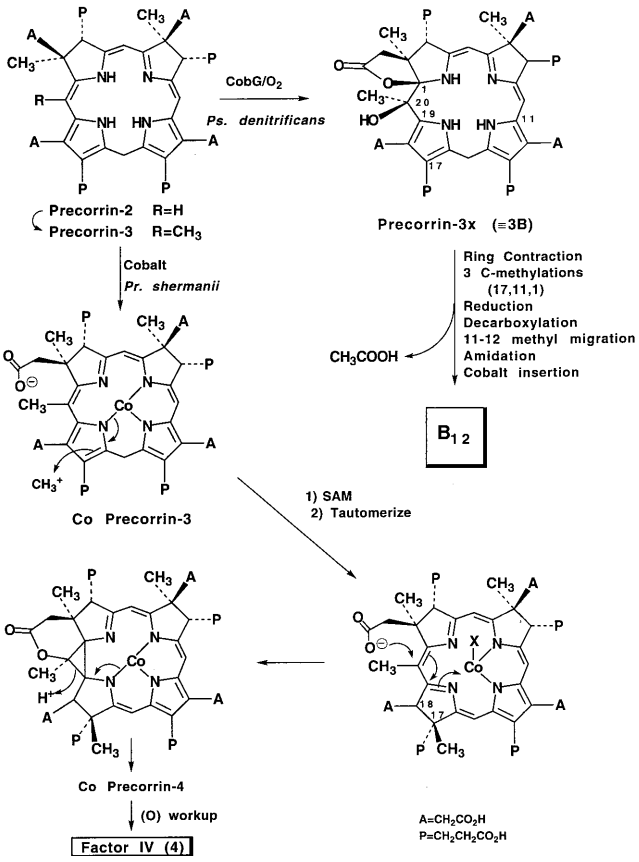
**ABSTRACT** **The structure of a novel tetradehydrocorrin, factor IV, isolated from *Propionibacterium shermanii* has been established by multidimensional NMR spectroscopy. Incorporation of radiolabeled factor IV into cobyrinic acid established the biointermediacy of this cobalt complex, whose structure has implications for the mechanisms of the anaerobic pathway to $B_{12}$.**

In the aerobic bacterium *Pseudomonas denitrificans*, the biosynthetic pathway to vitamin $B_{12}$, featuring an oxygen-requiring step, has been established (for reviews, see refs. 1 and 2), but it is now clear that a second, anaerobic route to corrin has existed in nature for *ca.* 4 billion years (2, 3). Thus, the obligate anaerobes, such as methanogenic bacteria, and the semianaerobic *Propionibacterium shermanii* are able to synthesize $B_{12}$ by processes that differ at several pivotal steps from the aerobic pathway. The *Ps. denitrificans* route uses a two-stage ring contraction sequence on the metal-free substrate precorrin-3, using $O_2$ (4) to fashion γ-lactone and tertiary hydroxyl functions at C-1 and C-20 in precorrin-3x (Scheme **I**) as a prelude to a pinacol-like rearrangement, whereas cobalt is inserted late, i.e., not until all *S*-adenosylmethionine (AdoMet)-derived methyl groups are in place and amidation of the carboxylates has begun (1, 2).

In contrast, it has been found (5) that in *Pr. shermanii* cobalt is inserted early into precorrin-3, which is ring-contracted without $O_2$ as cofactor (Scheme **I**), and at a later, unknown stage, a unique exchange of carbonyl oxygen at the C-27 (ring A) acetate occurs (6, 7); this is another event that is not paralleled in aerobic metabolism (8). In this report, we describe the isolation of a new $B_{12}$ intermediate from *Pr. shermanii* whose surprising structure provides circumstantial but compelling evidence for those mechanisms operating during anaerobic biosynthesis that mediate ring contraction and loss of the 2-carbon fragment from the western side.




Scheme **I**

## MATERIALS AND METHODS

**Preparation of Factor IV from epi-Factor II.** A cell-free extract of *Pr. shermanii* (from 50 g of wet cells) was prepared by sonication (50 min at 0–4°C) in phosphate buffer (pH 7.6; 50 ml). Glutathione (5 mg), ATP (22 mg), NAD (5 mg), NADH (10 mg), AdoMet (75 μmol), EDTA (3.5 μmol), $Co^{2+}$ (4 μmol), and $Co^{3+}$ (1.8 μmol) were added to the supernatant of the centrifuged suspension (17,000 × *g*; 25 min at O°C). For preparative isolation, 3-epi; 8-epi factor II (Fig. 1; 500 μmol) was added, and the incubation was carried out at 31°C for 16 hr. Sodium cyanide (5 mg) was then added, and the tetrapyrrolic mixture was adsorbed on DEAE–Sephadex A-25 (Pharmacia) followed by extraction and esterification by MeOH/$H_2SO_4$ (95:5) for 16 hr at 20°C. The mixture was extracted with $CH_2Cl_2$ and chromatographed on silica gel in the solvent mixture toluene/EtOAc/MeOH (150:96:4; vol/vol/vol) in the presence of NaCN. Rechromatography of the baseline fraction in $CH_2Cl_2$/MeOH (95:5) afforded factor IV methylester (8 nmol) ($R_f$ 0.46) λmax($CH_2Cl_2$)-760 (0.47); 702 (0.50) 486 (0.39) 387 (1.00) 341 (0.67) 299 (0.66) nm (rel. intensities) FABMS $C_{51}H_{63}N_4O_{16}$ Co: *m/z* 1046.

**Isolation of $^{13}$C-Enriched Factor IV.** The above incubation was carried out in the presence of [4-$^{13}$C]δ-aminolevulinic acid (ALA), [5-$^{13}$C] ALA, and $^{13}CH_3$-AdoMet to afford specimens of pure factor IV (200–250 μg), which were used for NMR structural determination by the methods previously described for precorrin-3x (8).

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Abbreviations: ALA, δ-aminolevulinic acid; AdoMet, *S*-adenosylmethionine.

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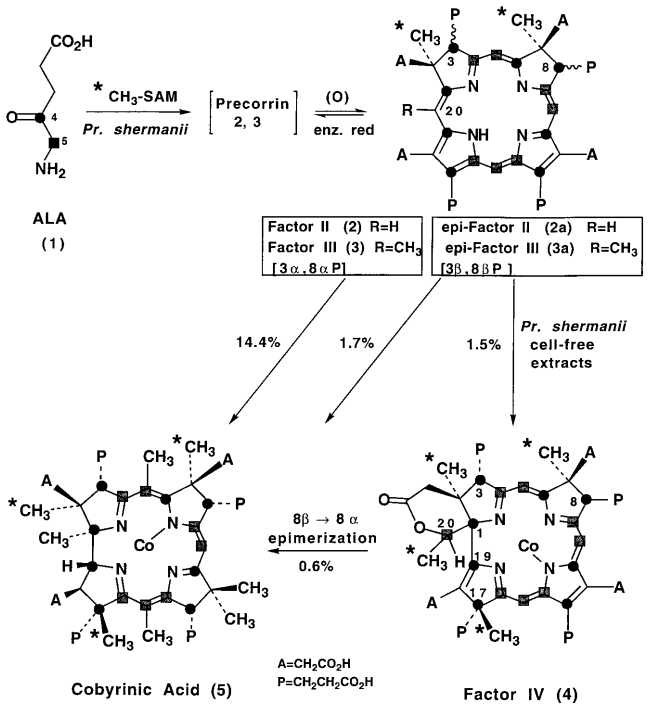



FIG. 1. Bioconversion (% radiochemical yields) of factors II and III, (and their 3 and 8 epimers) derived from labeled ALA and AdoMet and of factor IV into cobyrinic acid (**5**) using cell-free extracts of *Pr. shermanii*. Labeling patterns were derived from either stable isotopes (●, [4-$^{13}$C]ALA; ■, [5-$^{13}$C]ALA; ∗, [methyl-$^{13}$C]AdoMet) or radioisotopes (●, [4-$^{14}$C]ALA; ∗, [methyl-$^{3}$H]AdoMet) as described in the text. The intermediates are isolated as methyl esters and reincubated (after hydrolysis) in their oxidized forms (factors II–IV) but it is assumed that the biotransformations occur at the reduced (precorrin) level since precorrin-2 can be isolated anaerobically from *Pr. shermanii* (9).

**Bioconversion of Factor II, (3*β*,8*β*-epi) Factor II, and Factor IV to Cobyrinic Acid.** Doubly labeled factors were prepared as described previously (5) from [5-$^{14}$C]ALA and C$^{3}$H$_3$-AdoMet. Cobester was isolated from incubation of cell-free extract with the radiolabeled factors and chromatographed to constant radioactivity. The radio incorporations were determined by $^{14}$C/$^{13}$H scintillation counting, and the results are summarized in Fig. 1.

**NMR Correlations of Factor IV.** $^{13}$C-chemical shifts, heteronuclear multiple bond, and heteronuclear multiple quantum correlations were acquired exactly as described for the structure determination of precorrin-3x (8). The assignments are summarized in Table 1.

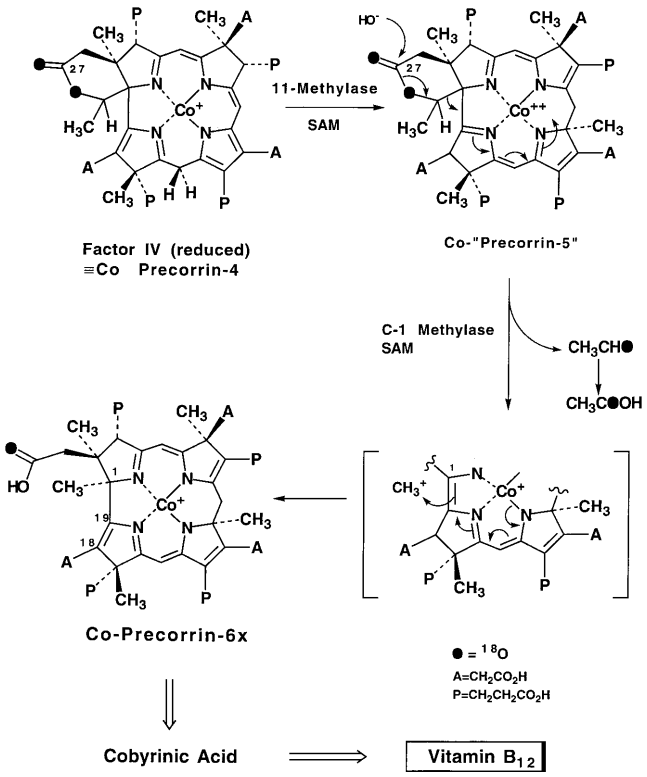
## RESULTS AND DISCUSSION

Factor IV (**4**) was isolated from cell-free extracts of *Pr. shermanii* by esterification (MeOH/$H_2SO_4$) and multiple thin-layer chromatography. The presence of four methyl groups was deduced by the $^{3}$H/$^{14}$C ratios of specimens derived from [*methyl*-$^{3}$H$_3$]-AdoMet and either $^{14}$C factor II (**2**) or $^{14}$C factor III (**3**) (Fig. 1, ∗ = $^{3}$H; ● = $^{14}$C). Mass spectral analysis of the esters prepared from both MeOH (*m/z* 1046) and EtOH (*m/z* = 1144) revealed that seven ester groups were present (M + 98 for the ethyl analog); the eighth carboxylic function was a δ-lactone (FTIR: 1740 cm-$^{1}$). When factor IV was isolated from incubations using [4-$^{13}$C]ALA (**1**) and [*methyl*-$^{13}$C]AdoMet as substrates, one-dimensional $^{13}$C-NMR analysis (see Table 1) of the resultant $^{13}$C-enriched heptamethyl ester (Fig. 1, factor IV **4**: A = $CH_2CO_2Me$; P = $CH_2CH_2CO_2Me$; ● = $^{13}$C) showed characteristic C-C coupling (*J* = 58 Hz) between C-19 and C-1, which is evidence that ring contraction had occurred, whereas coupling (*J* = 33 Hz) between one of the four AdoMet-derived methyl groups and a *propionate* terminus (δ 64.0) suggested C-methylation at C-17. The observation of coupled doublets (*J* = 38 Hz) at δ 81.9 and 18.8 ppm in factor IV enriched from [5-$^{13}$C]ALA and $^{13}$C-AdoMet (Fig. 1, ■ and ∗ = $^{13}$C) indicates that C-20 and its attached methyl group are still present in the molecule. Further NMR analyses (distortionless enhancement for polarization transfer, heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation) disclose that C-20 is appended to C-1, and bears a proton, a methyl group, and the oxygen terminus of the δ-lactone as the fourth substituent. The assignment of methyl resonances at δ 20.1 and δ 27.3 ppm to C-2 and C-7, respectively, reveals that while C-3 is in the natural (*α*) configuration, the C-8 propionate is *β*-oriented, thereby releasing the *γ*-effect on the adjacent C-7 methyl, a well-known phenomenon in such epimerizations (10, 11).

Table 1. $^{13}$C NMR assignments of factor IV heptamethyl ester in benzene-$d_6$

| Position enriched | δ$^{13}$C (*J*cc Hz) |
|---|---|
| From [5-$^{13}$C]ALA | |
| C4 | 178.0 (68) |
| C5 | 96.6 (69) |
| C9 | 177.5 (65) |
| C10 | 95.2 (65) |
| C14 | 151.8 (75) |
| C15 | 99.8 (75, 69) |
| C16 | 173.0 (69) |
| C20 | 81.9 (38) |
| From [4-$^{13}$C]ALA | |
| C1 | 83.4 (57.8) |
| C3 | 60.16 |
| C6 | 171.76 |
| C8 | 55.7 |
| C11 | 157.86 |
| C13 | 146.68 |
| C17 | 64.0 (33) |
| C19 | 151.4 (58.2) |
| Methyl groups from $^{13}CH_3$-AdoMet | |
| C2 | 20.13 |
| C7 | 27.27 |
| C17 | 21.26 (33) |
| C20 | 18.77 (38) |

Incubation of $^{14}$C-labeled factor IV [biosynthesized from a mixture of 3-epi and 8-epi factor II (**2a**) with *Pr. shermanii* extracts] affords cobyrinic acid (**5**, isolated as cobester; A = $CH_2CO_2Me$; P = $CH_2CH_2CO_2Me$) in 0.6% radiochemical yield [Fig. 1, (**4**) → (**5**)]. Epimerization at C-8 of factor II has obviously caused derailment, thereby allowing isolation of 8*β*-configured factor IV, which is partially reepimerized to the natural (3*α*, 8*α*) isomer to allow synthesis of (**5**), confirmed by conversion of a mixture of 3-epi and 8-epi factor II (**2a**) directly to cobyrinic acid (**5**) in a comparable 1.7% radiochemical yield, together with a substantial amount of 8-epi factor IV. Indirect evidence for inversion of stereochemistry at C-8 had previously been observed by the exchange of H for D at C-3, C-8, and C-13 when vitamin $B_{12}$ was isolated from *Pr. shermanii* cells grown in 50% $^{2}H_2O$ (12). Factor IV, whose absolute stereochemistry at C-2, C-7, and C-17 follows from bioconversion to cobyrinic acid, has been oxidized during isolation, and like factor II must be reduced by enzyme(s) present in the cell-free extract (1, 2, 9) to the precorrin-4 oxidation level (Scheme **II**) and then epimerized at C-8 to allow incorporation to proceed. It should be noted that precorrin-2 and -3 were first isolated in their oxidized forms, factors II and III, respectively, and are reduced back to the corresponding precorrins by reductive enzyme(s)




Scheme **II**

present in the cell-free extracts of *Pr. shermanii* and thus reenter the biosynthetic pathway (9).

A mechanism for the formation of factor IV is proposed in Scheme **I** and features δ-lactone formation at C-20 that delivers the oxygen required for subsequent loss of the "$C_2$" unit. Formation of a δ-lactone from the ring A acetate to the C-20 position was first promulgated by Eschenmoser (13) as a means of functionalizing C-20 prior to ring contraction and as part of a scaffolding device for the ring contraction process. The discovery of the "aerobic" mechanism in which molecular oxygen serves as the source of the C-20 hydroxyl in precorrin-3x (=3B) (Scheme **I**) led Eschenmoser to suggest recently (14) that involvement of the δ-lactone, however attractive as a chemical model, would probably require an oxidative step. The new structure of factor IV, together with the experimental demonstration of the transfer of $^{18}O$ from the ring A carboxylate to the acetic acid isolated in the anaerobic pathway (15) and the loss of $^{18}O$ from the same carboxylate function (6, 7), now provide strong support for the renaissance of the nonoxidative "δ-lactone" hypothesis, although not quite in its original form. The ring contraction takes place by a mechanism that may involve a cyclopropane intermediate followed by ring opening to forge the A-D ring linkage, leaving the pendant C-20 at the oxidation level of acetaldehyde. Thirty years ago, before the origin (AdoMet) of the C-1 methyl group of $B_{12}$ was discovered, Eschenmoser and colleagues (16, 17) had envisaged a cyclopropane intermediate formed by proton catalyzed reduction at the 20-meso position as part of the ring contraction process. Again, Eschenmoser (14) has referred to the "death of this cherished hypothesis" when the origin of the C-1 methyl group of $B_{12}$ was found to be methionine (2) rather than the C-20 meso carbon. The biosynthesis of factor IV may indeed involve just such a cyclopropane intermediate (Scheme **I**), which subsequently loses C-20 as acetaldehyde (→ acetic acid), thereby reviving one of the earliest concepts for ring contraction. Several alternatives remain, including proton catalyzed ring closure of a seco-corrinoid (18, 19). Thus, in cobalt precorrin-4 (Scheme **II**) the ring contraction is orchestrated not by molecular oxygen, but by internal delivery of oxygen functionality that in turn could be mediated by remote interaction of cobalt with the carboxylate function in ring A and/or addition of the fourth C-methyl group in ring D at position 17 or 18. The latter position is favored stereoelectronically but would require subsequent migration of the methyl to C-17, just as is found for C-11 → C-12 methyl shift in the aerobic pathway. Subsequent hydrolysis of the δ-lactone rationalizes the specific exchange of $^{18}O$ at C-27 (6, 7) and the transfer of $^{18}O$ to C-20 (15) and is coupled with retro-aldol release of acetaldehyde [from the putative Co-precorrin-5 (Scheme **II**)]. The excision of acetaldehyde requires revision of a previous mechanism (20, 21) based on loss of acetic acid, and would also necessitate a subsequent enzyme-catalyzed oxidation of the liberated acetaldehyde to acetic acid, which was isolated from *Pr. shermanii* extracts fed with $^{14}C$-labeled precursors (20, 21). Conclusive evidence for this scenario is provided in the accompanying paper by Wang *et al.* (22).

The suggested anaerobic pathway to $B_{12}$ is shown in Scheme **II** where it is assumed that the two pathways converge at precorrin -6x(23). Verification must await the discovery of the appropriate biosynthetic enzymes by analysis of a genomic library of *Pr. shermanii* from which the first methyl transferase (CobA) has recently been isolated (24). The unique structure of factor IV adds strength to the postulate that all of the anaerobic intermediates from precorrin -2 onward are cobalt complexes (5, 25) and explains why attempts to elicit the appropriate enzyme activities from cloned *Salmonella* genes (26) that are responsible for corrin synthesis have so far been unsuccessful using cobalt-free substrates, although some crossover between the aerobic, metal-free intermediates precorrin -6x and -8x was observed when these were incorporated (albeit in low yield) into cobyrinic acid in cell-free extracts of *Pr. shermanii* (23).

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1. Blanche, F., Cameron, B., Crouzet, J., Debussche, L., Thibaut, D., Vuilhorgne, M., Leeper, F. J. & Battersby, A. R. (1995) *Angew. Chem. Intl. Ed. Engl.* **34,** 383–411.
2. Scott, A. I. (1994) *Tetrahedron* **50,** 13315–13334.
3. Scott, A. I. (1994) *Heterocycles* **39,** 471–476.
4. Spencer, J. B., Stolowich, N. J., Roessner, C. A., Min, C. & Scott, A. I. (1993) *J. Am. Chem. Soc.* **115,** 11610–11611.
5. Müller, G., Zipfel, F., Hlineny, K., Savvidis, E., Hertle, R., Traub-Eberhard, V., Scott, A. I., Williams, H. J., Stolowich, N. J., Santander, P. J., Warren, M. J., Blanche, F. & Thibaut, D. (1994) *J. Am. Chem. Soc.* **113,** 9893–9895.
6. Scott, A. I., Stolowich, N. J., Atshaves, B. P., Karuso, P., Warren, M. J., Williams, H. J., Kajiwara, M., Kuramaya, K. & Okazaki, T. (1991) *J. Am. Chem. Soc.* **113,** 9891–9892.
7. Vishwakarma, R. A., Balachandran, S., Alanine, A. I. D., Stamford, N. P. J., Kuichi, F., Leeper, F. J. & Battersby, A. R. (1993) *J Chem. Soc. Perkin Trans. 1* 2893–2898.
8. Stolowich, N. J., Wang, J., Spencer, J. B., Roessner, C. A., Santander, P. J. & Scott, A. I. (1996) *J. Am. Chem. Soc.* **118,** 1657–1662.
9. Battersby, A. R., Frobel, K., Hammerschmidt, F. & Jones, C. (1982) *J. Chem. Soc. Chem. Commun.* 455–458.
10. Scott, A. I., Townsend, C. A. & Cushley, R. J. (1973) *J. Am. Chem. Soc.* **95,** 5759–5761.
11. Battersby, A. R., McDonald, E., Neier, R. & Thompson, M. (1979) *J. Chem. Soc. Chem. Commun.* 960–962.
12. Scott, A. I., Kajiwara, M. & Santander, P. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6616–6620.
13. Eschenmoser, A. (1988) *Angew. Chem. Int. Ed. Engl.* **27,** 5–39.
14. Eschenmoser, A. (1994) *CIBA Found. Symp.* **180,** 310–323.
15. Arigoni, D. (1994) *CIBA Found. Symp.* **180,** 280–281.
16. Wehrli, P. (1967) Ph.D. dissertation (Eidgenössiche Technische Hochschule, Zurich).

17. Muller, P. M., Farooq, S., Hardegger, B., Salmond, W. S. & Eschenmoser, A. (1973) *Angew. Chem. Int. Ed. Engl.* **12,** 914–916.
18. Yamada, Y., Miljkovic, D., Wehrli, P., Golding, B., Löliger, P., Keese, R., Müller, K. & Eschenmoser, A. (1969) *Angew. Chem. Int. Ed. Engl.* **8,** 343–348.
19. Ofner, S., Rasetti, V., Zehnder, B. & Eschenmoser, A. (1981) *Helv. Chim. Acta* **64,** 1431–1443.
20. Mombelli, L., Nussbaumer, C., Weber, H., Müller, G. & Arigoni, D. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 9–10.
21. Battersby, A. R., Bushell, M. J., Jones, C., Lewis, N. G. & Pfenninger, A. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 13–15.
22. Wang, J., Stolowich, N. I., Santander, P. J. & Scott, A. I. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14320–14322.
23. Blanche, F., Thibaut, D., Debussche, L., Hertle, R., Zipfel, F. & Müller, G. (1993) *Angew. Chem. Int. Ed. Engl.* **32,** 1651–1653.
24. Sattler, I., Roessner, C. A., Stolowich, N. J., Hardin, S. H., Harris-Haller, L. W., Yokubaitis, N. T., Murooka, Y., Hashimoto, Y. & Scott, A. I. (1995) *J. Bacteriol.* **177,** 1564–1569.
25. Balachandran, S., Vishwakarma, R. A., Monaghan, S. M., Prelle, A., Stamford, N. P. J., Leeper, F. J. & Battersby, A. R. (1994) *J. Chem. Soc. Perkin Trans. 1* 487–491.
26. Roessner, C. A., Warren, M. J., Santander, P. J., Atshaves, B. P., Ozaki, S.-I., Stolowich, N. J., Iida, K. & Scott, A. I. (1992) *FEBS Lett.* **301,** 73–78.