Synthesis and Biological Activity of 9-Mercaptodethiobiotin—a Putative Biotin Precursor in *Escherichia coli*

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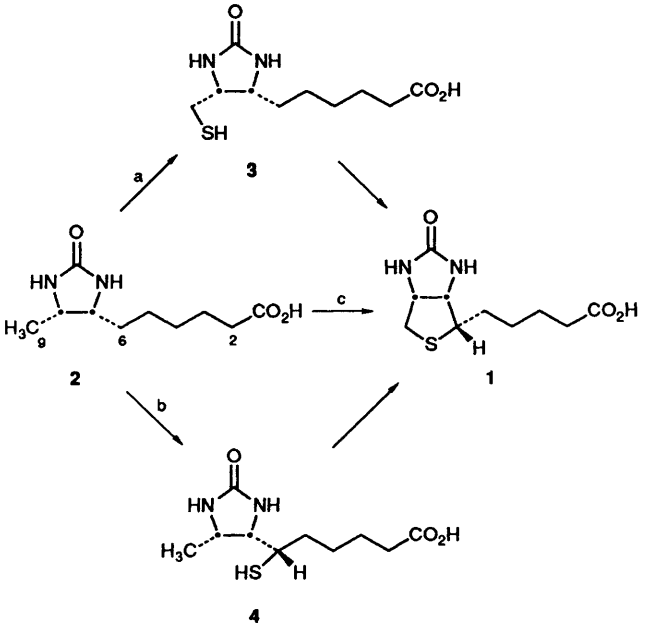
A total synthesis of (±)-9-mercaptodethiobiotin **3** *via* the aldehyde **9** is described. Compound (±)-**3** does not function as a biotin replacement factor for an *E. coli* mutant (SA291) lacking the entire biotin synthesis operon (*bio*ABFCD$^-$) but supports growth of an *E. coli bio*A mutant. Compound (±)-**3** also supports growth of transformed cells of SA291 carrying a plasmid encoding the *E. coli* biotin synthase (*bio*B) gene indicating that the compound may be able to substitute for dethiobiotin **2** as a substrate for biotin synthase.

The coenzyme biotin **1** is a commercially important vitamin which is produced only in minute amounts from biological sources. The mechanistic aspects of the role of biotin as the carrier of carbon dioxide in enzymatic carboxylation and transcarboxylation reactions continues to be the subject of investigation.[1] Perhaps the most interesting step in the pathway of biotin biosynthesis is the final step which involves the introduction of sulfur between C-6 and C-9 of the dethiobiotin skeleton **2**. Parry[2] has shown that the substitution of sulfur at C-6 involves loss of the *pro-S* hydrogen, resulting in retention of configuration at this position. In contrast, insertion of sulfur at the C-9 position of **2** appears to involve racemisation of the methyl group hydrogens.[3] In *E. coli* and *Bacillus sphaericus* the sulfur insertion reaction is apparently catalysed by a single enzyme, biotin synthase, which is encoded by the *bio*B gene of the biotin operon.[4,5] While the identity of the cellular sulfur donor is at present unknown it can be envisaged that the reaction catalysed by biotin synthase proceeds by one of three mutually exclusive pathways—involving either (a) the intermediacy of the primary thiol **3**; (b) the intermediacy of the secondary thio **4**; or (c) a 'concerted' reaction in which no enzyme-free intermediate is involved (Scheme 1). Recently

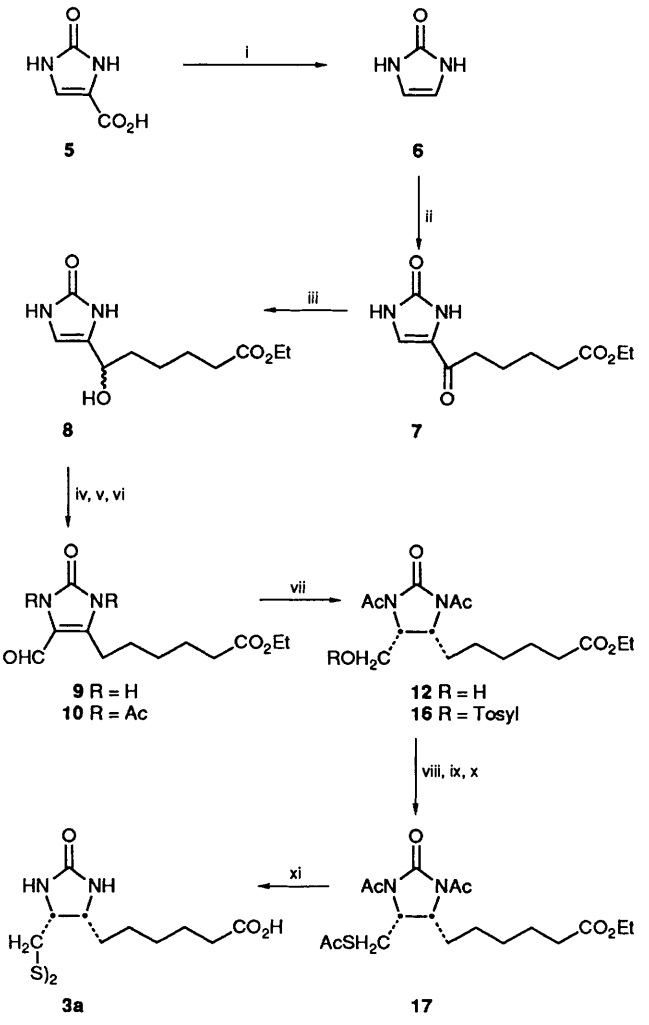


Scheme 1

Marquet and her co-workers have reported a synthesis of the methyl ester of the primary thiol **3** from a chiral starting material and have cited preliminary evidence for the incorporation of **3** into biotin by cells of *B. sphaericus*.[6] In this paper we describe the synthesis of (±)-**3** using a different approach and evidence from studies with *bio*$^-$ mutants and transformed strains of *E. coli* which support a precursorial role for **3**.

Synthesis of the disulfide dimer of (±)-9-mercaptodethiobiotin, **3a**, was carried out as outlined in Scheme 2. Synthesis of the key intermediate aldehyde **9** was achieved in a straightforward manner by a refinement of the route used by Zavylov.[7] Thus condensation of (±)-tartaric acid and urea afforded the carboxylic acid **5** which was decarboxylated, by treatment with K_2CO_3, to give imidazolin-2-one **6**.[8] Acylation of **6** with ethyl 5-chloroformylpentanoate in the presence of $AlCl_3$ afforded the ketone **7** which was reduced with $NaBH_4$ to give an epimeric mixture of the corresponding allylic alcohols **8**. Condensation of **8** with paraformaldehyde and piperidine gave a Mannich base adduct which was hydrolysed with aqueous acetic acid to afford **9**.

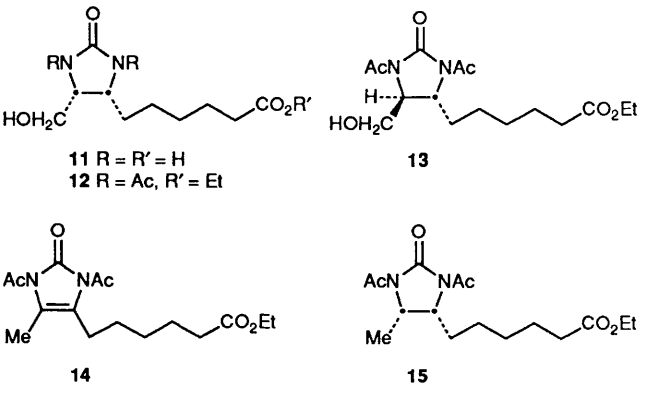
To render the double bond of the imidazolinone ring susceptible to catalytic hydrogenation, the aldehyde **9** was acetylated to give the *N,N'*-diacetylated product **10**. In an earlier study of the synthesis of (±)-9-hydroxydethiobiotin **11**, Frappier *et al.*[9] have shown that catalytic reduction of **11** over 10% palladium on charcoal in dioxane affords a 60:40 mixture of the *cis*- and *trans*-9-oxo-*N,N'*-diacetyl derivatives, **12** and **13**, presumably due to epimerisation of the C-8 centre after reduction. To circumvent the epimerisation at the C-8 centre, which was a major drawback in the earlier synthesis, we carried out the reduction under conditions where hydrogenolysis of the aldehyde function was slightly favoured over reduction of the imidazolinone double bond. Thus hydrogenation of **10** over pre-reduced Adam's catalyst in glacial acetic acid gave ethyl (±)-*N,N'*-diacetyl-9-hydroxydethiobiotin **12** as the major product accompanied by minor amounts of the easily separated *N,N'*-diacetyl derivatives **14** and **15**. The observation of a 10.1 Hz coupling between 7-H and 8-H in the ^{1}H NMR of **12** confirmed the *cis* configuration at the 7 and 8 positions. Under these conditions no significant amounts of products with *trans*-configuration at the 7 and 8 position were detected. Our initial approach to the introduction of the C-9 thiol function involved tosylation of the alcohol **12** to give the ester **16**, followed by substitution of the tosyl group with the anion of toluene-α-thiol.[10] However, the latter reaction afforded a plethora of products. Displacement of the tosyl group could, however, be



Scheme 2 i, K_2CO_3; ii, $Cl{\cdot}CO(CH_2)_4CO_2Et$, $AlCl_3$, $C_6H_5NO_2$; iii, $NaBH_4$, MeOH; iv, $(HCHO)_n$, piperidine, MeOH; v, HOAc; vi, Ac_2O; vii, H_2, PtO_2, HOAC; viii, Tosyl Cl, pyr; ix, $KSCOCH_3$, DMF; x, NaOH, MeOH

achieved cleanly using sodium thioacetate to afford the 9-thioacetyl derivative **17** in 82% yield. Hydrolysis of **17** in aqueous alkali, followed by purification of the product by cation exchange chromatography afforded the dimer **3a**. Confirmation of the identity of the product was evinced from mass spectrometry [*m/z* 491.1998; $C_{20}H_{35}N_4O_6S_2$, $(M + 1)^+$] and the appearance of a methylene resonance at 43.0 ppm in the ^{13}C NMR spectrum indicative of a disulfide methylene carbon.[11] Desulfurisation of **3a** with Raney nickel afforded (±)-dethiobiotin **2** confirming the relative stereochemistry at the C-7 and C-8 positions.

Reduction of **3a** to the primary thiol **3** could be achieved by treatment with dithiothreitol (DTT) in water. In the ^{13}C NMR DEPT spectrum the methylene resonance at 43.0 ppm was replaced by a peak at 26.2 ppm indicative of a CH_2SH function.[11] However, it was not possible to isolate the monomeric thiol in a pure form; attempts to separate the monomeric and dimeric forms from the reducing agent, even in degassed solvents, gave mixtures of **3** and **3a**.*



Evidence for the possible role of (±)-9-mercaptodethiobiotin **3** as an intermediate in biotin formation in *E. coli* is suggested by growth experiments on *bio*-mutants. Even at very high concentrations (>1 mmol dm^{-3}) the thio does not support growth of SA291, a *bio*ABFCD deletion mutant which lacks all of the biotin synthesis genes and requires biotin for growth,[4a] indicating that the compound itself does not substitute for biotin as a coenzyme. However, at concentrations above 4 μmol dm^{-3} it supports normal growth of an *E. coli*, *bio*A mutant (strain 6435), which lacks functional 7,8-diaminopelargonate synthase activity and thus requires 7,8-diaminopelargonate, dethiobiotin or biotin for growth. At this concentration (±)-**3** also supported growth of cells of the *bio*ABFCD deletion strain SA291 transformed with a pKK223 derived plasmid† carrying a functional *E. coli* biotin synthase gene under control of a *tac* promoter (pK*bio*$^-$B) and which is thus able to grow in dethiobiotin supplemented minimal medium.

While this evidence suggests that the primary thiol **3** may act as a biosynthetic intermediate between dethiobiotin **2** and biotin **1** in *E. coli* it should be noted that it is also consistent with an alternative hypothesis that **3** could merely act as an abnormal precursor of dethiobiotin *in vivo*. That is, that **3** could be catabolised by an, as yet, uncharacterised desulfurisation process to give sufficient dethiobiotin for growth of biotin synthase competent cells.

Further studies on the mechanism of the conversion of **2** into **1** are underway.

Experimental

NMR spectra were recorded on Bruker WP80, WP200 and WM360 spectrometers; *J* values in Hz. Mass spectra were measured using a Kratos ZAB spectrometer. IR spectra were recorded on a Perkin-Elmer X98 spectrophotometer. M.p.s were determined on a Reichert hot-stage apparatus and are uncorrected.

4-(5-*Ethoxycarbonyl*-1-*oxovaleryl*)*imidazolin*-2-*one* **7**.—A ground mixture of (±)-tartaric acid (42 g) and urea (13 g) was slowly added to stirred conc. H_2SO_4 (100 cm^3) the rate being adjusted so that the temperature did not exceed 65 °C without external cooling. The resultant mixture was heated at 80 °C for 1 h, cooled to 20 °C and poured on crushed ice (1 kg). The resulting precipitate was filtered off and washed with water (0.5 dm^3) and acetone (100 cm^3) to afford 4-*carboxyimidazolin*-2-*one* **5** (19 g, 53%), m.p. 226–230 °C (lit.,[7] 232–235 °C); $\delta_H([^2H_6]DMSO)$ 9.8 (3 H, br s) and 7.15 (1 H, s); ν_{max}(Nujol)/cm^{-1} 3200, 1759, 1670 and 1600. A solution of compound **5** (27 g) in aq. K_2CO_3 (0.7 mol dm^{-3}; 300 cm^3) was

* In biological experiments **3a** was administered to cells in 5 mmol dm^{-3} DTT, *i.e.* principally as the monomer **3**. In the reducing environment of the cytosol (see, for example. B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts and J. D. Watson in *Molecular Biology of the Cell*, 2nd edn., 1989, Garland, London, p. 445) we would expect the monomeric form to be predominant.

† Details of the construction of plasmids containing the biotin synthase (*bio*B) gene and the *bio*ABFCD gene cluster will be described elsewhere.

heated at reflux for 4 h, charcoal (1 g) added, and the solution refluxed for a further 0.5 h. The hot solution was filtered, evaporated under reduced pressure to *ca.* 80 cm^3 and the resultant precipitate washed with acetone (30 cm^3) and ether (20 cm^3 × 2) to give *imidazolin-2-one* **6** (14 g, 75%), m.p. 238–240 °C (lit.,[8] 240–241 °C); δ_H[$^2H_6$]DMSO) 9.71 (2 H, br s) and 6.23 (2 H, br s); ν_{max}(Nujol)/cm^{-1} 3120, 1604 and 1575. Powdered $AlCl_3$ (72 g) was added over 1 h to a stirred, cooled solution of compound **6** (13 g) in nitrobenzene (130 cm^3) at 10 °C. The solution was stirred for a further 0.5 h and ethyl 5-chloroformylpentanoate[7] (35 g) slowly added. The stirred solution was heated at 65 °C for 4 h, poured into ice–1 mol dm^{-3} aq. Na_2CO_3 (1:1; 600 cm^3), mixed with ether (500 cm^3) and filtered. The precipitate was dissolved in hot water (20 cm^3), treated with charcoal (0.1 g), filtered and cooled to afford **7** (15.2 g, 43%) as colourless needles, m.p. 236–240 °C (lit.,[7] 239–242 °C); δ_H([$^2H_6$]DMSO) 10.39 (2 H, br s, NH), 7.59 (1 H, s, 5-H), 4.04 (2 H, q, *J* 7.0, OCH_2CH_3), 2.64 (2 H, br t, *J* 7, $=CCH_2$–), 2.37 (2 H, br t, *J* 7, CH_2CO_2Et), 1.53 (4 H, m, CH_2CH_2), and 1.17 (3 H, t, *J* 7.0, CH_2CH_3).

Ethyl 9-*Oxo*-7,8-*didehydrodethiobiotin* **9**.—Powdered $NaBH_4$ (2.7 g) was slowly added to a stirred methanolic solution of compound **7** (12 g, 250 cm^3) over 5 min and the solution was stirred at room temperature for 2.5 h, filtered, and evaporated to dryness. The residue was washed successively with water (20 cm^3), acetone (20 cm^3) and ether (20 cm^3) and crystallised from EtOAc–EtOH to afford a mixture of the 6*R* and 6*S* alcohols **8** (7.5 g, 62%), m.p. 170–173 °C. The mixture of alcohols was dissolved in MeOH (120 cm^3) and paraformaldehyde (1.1 g) and piperidine (3.1 cm^3) were added; the mixture was then heated at reflux for 8 h. Removal of MeOH and piperidine under reduced pressure afforded the crude product as a brown solid (7.2 g) which was dissolved in glacial HOAc (80 cm^3) and heated at 60 °C for 10 h. Evaporation of the solvent gave an oil which was separated by flash column chromatography on SiO_2 using EtOAc followed by crystallisation from EtOAc to give **9** (2.5 g, 46%), m.p. 143–145 °C (lit., 145–146 °C); δ_H($CDCl_3$) 11.57, 9.87 (2 H, br s, NH), 9.43 (1 H, s, CHO), 4.10 (2 H, q, *J* 7.0, CH_2CH_3), 2.77 (2 H, t, *J* 7.0, 6-H), 2.28 (2 H, t, *J* 7.0, 2-H), 1.49 (6 H, m, $CH_2CH_2CH_2$), 1.22 (3 H, t, *J* 7.0, CH_2CH_3); ν_{max}(Nujol)/cm^{-1} 1730, 1710 and 1668.

Ethyl (±)-N,N′-*Diacetyl*-9-*hydroxydethiobiotin* **12**.—A solution of compound **9** (10 g) in AcO_2 (100 cm^3) was heated at reflux for 1 h and evaporated under reduced pressure to afford an oil which was fractionated by flash column chromatography on SiO_2, using EtOAc–hexanes (2:3) as eluent, to give the *N*,*N*′-diacetyl derivative **10** (11.5 g, 86%) as a colourless oil; δ_H($CDCl_3$) 10.13 (1 H, s, CHO), 4.22 (2 H, q, *J* 7.0, CH_2CH_3), 3.28 (2 H, t, *J* 7.0, 6-H), 2.65 (6 H, s, $NCOCH_3$), 2.40 (2 H, t, *J* 7.0, 2-H), 1.7 (6 H, m, $CH_2CH_2CH_2$) and 1.31 (3 H, t, *J* 7.0, CH_2CH_3); ν_{max}(film)/cm^{-1} 1825, 1735 and 1690.

A stirred suspension of PtO_2 (1.5 g) in glacial HOAc (10 cm^3) was pre-reduced with H_2 at ambient pressure for 1.5 h and a solution of **10** (2.3 g) in HOAc added. After 3 h the catalyst was filtered off and washed with HOAc (6 cm^3) and the filtrate and washings were evaporated under reduced pressure to afford an oil. This was fractionated by flash column chromatography on SiO_2, using 10–100% EtOAc–hexanes to afford the products **14** (0.4 g, 17%), **15** (0.24 g, 11%) and **12** (0.9 g, 40%) as colourless oils. Ethyl *N*,*N*′-diacetyldidehydrodethiobiotin **14**; δ_H($CDCl_3$) 3.95 (2 H, q, *J* 7.0, CH_2CH_3), 2.55 (2 H, m, 6-H), 2.46 (6 H, s, $NCOCH_3$), 2.16 (2 H, br t, *J* 7, 2-H), 1.6–1.3 (6 H, br m, $CH_2CH_2CH_2$) and 1.09 (3 H, t, *J* 7.0, CH_2CH_3); δ_C($CDCl_3$, DEPT) 60.06, 42.35, 33.76, 26.17, 25.51, 24.42, 24.10, 23.60, 14.01 and 11.99; ν_{max}(film)/cm^{-1} 1750, 1735 and 1705.

Ethyl (±)-*N*,*N*′-diacetyldethiobiotin **15**; δ_H($CDCl_3$) 4.23 (2 H, m, 7, 8-H), 3.95 (2 H, q, *J* 7.0, CH_2CH_3), 2.37, 2.36 (6 H, 2 s, $NCOCH_3$), 2.16 (2 H, br t, *J* 7, 6-H) and 1.8–1.1 (11 H, m); δ_C($CDCl_3$, DEPT) 54.62, 51.16, 50.29, 33.58, 28.74, 26.71, 25.09, 24.98, 24.77, 24.38 and 13.49; ν_{max}(film)/cm^{-1} 1755, 1735 and 1695.

Ethyl (±)-*N*,*N*′-diacetyl-9-hydroxydethiobiotin **12**; δ_H($CDCl_3$) 4.21 (1 H, dt, *J* 10.0, 5.1, 8-H), 3.98 (5 H, m), 3.12 (2 H, t, *J* 5.1, 9-H), 2.42 (2 H, m, 6-H), 2.36, 2.32 (6 H, 2 s, $NCOCH_3$), 2.13 (2 H, br t, *J* 7, 2-H), 1.4–1.2 (6 H, m) and 1.17 (3 H, t, *J* 7.0, CH_2CH_3); ν_{max}(film)/cm^{-1} 3440, 1750, 1735 and 1695.

Ethyl (±)-N,N′-*Diacetyl*-9-*thioacetyldethiobiotin* **17**.—A solution of tosyl chloride (0.87 g) in dry pyridine (3 cm^3) was slowly added to a stirred solution of compound **12** (0.4 g) in pyridine (5 cm^3) at −5 °C over 5 min. Solid diaminopyridine (1 mg) was added and the mixture allowed to come to room temperature; it was then stirred for a further 72 h. The mixture was poured on crushed ice (20 cm^3), 1 mol dm^{-3} aq. HCl added (5 cm^3) and the whole extracted with ether (20 cm^3 × 4). The ethereal extract was washed with water (20 cm^3), dried ($MgSO_4$) and evaporated to afford an oil (0.52 g) which was fractionated by flash column chromatography on SiO_2, employing a 10–60% EtOAc–hexanes gradient, to give the tosylate **16** (0.32 g, 55%) as a colourless waxy solid: δ_H($CDCl_3$) 7.68, 7.32 (4 H, ABA′B′, *J* 8.3, ArH), 4.26 (1 H, dd, *J* 10.0, 7.0, 8-H), 4.07 (4 H, m, 9-H, CH_2CH_3), 4.00 (1 H, dd, *J* 10.0, 6.0, 7-H), 2.52 (2 H, m, 6-H), 2.42, 2.41 (6 H, 2 s, $NCOCH_3$), 2.24 (2 H, m), 1.5 (4 H, m) and 1.20 (3 H, t, *J* 7.0, CH_2CH_3); ν_{max}(film)/cm^{-1} 1760, 1732, 1700, 1365 and 1185. To a stirred solution of compound **16** (0.31 g) in dry DMF (2 cm^3) at ambient temperature under N_2, a solution of $KSCOCH_3$ (0.11 g) in DMF (2 cm^3) was added over 0.5 h. The mixture was stirred for 20 h, after which the DMF was removed under reduced pressure and the residue dissolved in ether (30 cm^3). The extract was sequentially washed with water (10 cm^3 × 2), saturated brine (10 cm^3) and water (10 cm^3), dried ($MgSO_4$) and evaporated to afford a residue which was subjected to flash column chromatography on SiO_2, eluting with a 10% EtOAc–hexanes to EtOAc gradient, to afford **17** (0.2 g, 83%) as a colourless oil; δ_H($CDCl_3$) 4.15 (1 H, m, 8-H), 3.88 (1 H, dd, *J* 9.0, 3.5, 7-H), 4.06 (2 H, q, *J* 7.0, CH_2CH_3), 3.11 (2 H, AB of ABX, J_{AB} 10.0, J_{AX} 8.0, 9-H), 2.40, 2.39 (6 H, 2 s, $NCOCH_3$), 2.25 (3 H, s, $SCOCH_3$), 2.19 (2 H, br t, *J* 7, 2-H), 1.5 (8 H, m) and 1.22 (3 H, t, CH_2CH_3); δ_C($CDCl_3$) 173.4, 170.1, 169.7, 151.3 (CO), 60.1 (OCH_2CH_3), 54.8, 54.3 (C-7, 8), 33.5 (C-9), 32.2 (C-2), 30.7 (C-6), 30.2 ($SCOCH_3$), 28.4, 24.4, 23.8 (C-3, 4, 5), 24.1, 24.0 ($NCOCH_3$) and 14.0 (CH_2CH_3); ν_{max}(film)/cm^{-1} 1755, 1750, 1735, and 1700; *m*/*z* (EI) 400.1515 (M^+, $C_{18}H_{28}N_2O_6S$ requires 400.1516).

(±)-9-*Mercaptodethiobiotin Dimer* **3a**.—A methanolic solution of compound **17** (100 mg, 0.5 cm^3) was slowly added to 1 mol dm^{-3} aq. NaOH (3 cm^3) and the solution stirred for 1.5 h at room temperature. The pH was adjusted to 8 with 1 mol dm^{-3} aq. HCl and the solution applied to a column of AG50 × 2 cation exchange resin (1 × 20 cm, 200 mesh, H^+ form). The column was washed with water (30 cm^3) and eluted with EtOH–H_2O–NH_4OH (5:3:2). Fractions (1 cm^3) giving a positive nitroprusside test were pooled and lyophylised to give the dimer **3a** (36 mg, 60%) as a colourless hygroscopic solid; δ_H([$^2H_5$]pyridine) 4.63 (1 H, m, 8-H), 4.42 (1 H, m, 7-H), 3.6 (2 H, br m, 9-H), 2.95 (2 H, br t, *J* 7, 6-H), 2.25 (2 H, br t, *J* 7, 2-H) and 1.8 (6 H, m); δ_C(D_2O–H_2O, pH 8.5) 183.9 (C-1), 164.5 (NCON), 57.3, 57.1 (C-7, 8), 43.0 (C-9), 37.6 (C-2), 34.8, 28.6, 25.8, 24.0 (C-3, 4, 5, 6); ν_{max}(Nujol)/cm^{-1} 3330, 3300, 1710 and 1580; *m*/*z* (FAB/glycerol) 491.1998 [$(M + 1)^+$, $C_{20}H_{35}N_4O_6S_2$ requires 491.1998].

Growth of E. coli *Mutants*.—*E. coli* 6435 and SA291 strains

were maintained on minimal medium[12] containing 10 μg/cm^3 biotin. SA291 was transformed with pK *bio*$^-$B using standard $CaCl_2$ transformation techniques[12] and transformants selected and maintained by growth on M9 minimal medium containing 50 μg/cm^3 ampicillin.

Acknowledgements

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