Pergamon

Bioorganic & Medicinal Chemistry, Vol. 4, No. 7, pp 1065–1075, 1996
Copyright © 1996 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
PII: S0968-0896(96)00100-9
0968-0896/96 $15.00 – 0.00

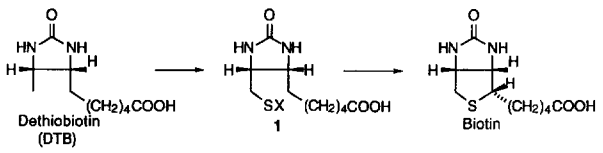
Synthesis and Biochemical Properties of *Z*- and *E*-4,5-Dehydrodethiobiotin

Eric Jestin, François Moreau, Dominique Florentin and Andrée Marquet*
Laboratoire de Chimie Organique Biologique, Université P. et M. Curie, URA CNRS 493, 4, place Jussieu, 75252 Paris Cedex 05, France

Abstract—Radical species are postulated intermediates in the formation of the carbon–sulfur bonds of biotin. It was of interest to examine the behaviour of unsaturated analogues which should give rise to allylic radicals. The two isomers of 4,5-dehydrodethiobiotin have been synthesized and labelled with ^{14}C on their carboxylic acid group. When incubated with an in vitro system capable of transforming dethiobiotin into biotin, they covalently label biotin synthase. Copyright © 1996 Elsevier Science Ltd

Introduction

The last step of biotin biosynthesis, namely the introduction of sulfur into dethiobiotin (DTB), has long raised a very puzzling problem, mainly because there has been no active cell-free system available. We have, however, been able to show[1] using intact cells, that compound **1** (X non-identified) was a likely intermediate formed through a completely new type of mechanism (Scheme 1).



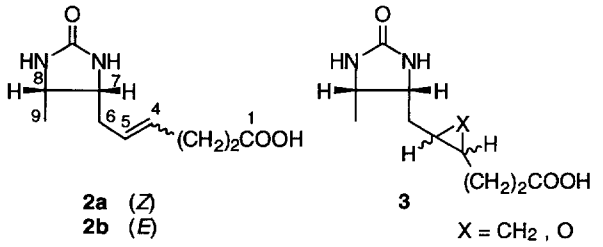
Scheme 1.

It is reasonable to assume that the activation of positions 6 and 9 is achieved via the formation of radicals. Evidence for the occurrence of an intermediate radical at C-9 arose from stereochemical studies carried out by Marti[2] who observed a complete racemization with DTB containing a chiral methyl group. To check the formation of a radical at position 6 in the intact cells, we decided to introduce a cyclopropyl or an epoxide group in the side chain of DTB (compounds **3**). It was also of interest to examine the behaviour of the ethylenic precursors **2** (Scheme 2).

More recently, active in vitro systems have been developed with *Escherichia coli*[3–5] and *Bacillus sphaericus*.[6,7] Biotin synthase of *E. coli*[4] and *B. sphaericus*[11] have been purified. The first one has been shown to contain a [2Fe–2S] cluster[4] by UV-visible and electron paramagnetic resonance (EPR) spectroscopy. Both enzymes have similar UV-visible spectra, but the EPR studies of *B. sphaericus* are not yet available. In vitro activity is observed with an assay mixture containing besides biotin synthase, *S*-adenosylmethionine (AdoMet), β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH), an electron transport system and other protein(s) and/or cofactors brought as a cell-free extract of *bio B* (−) strains of *E. coli*[4,5] or wild strains of *B. sphaericus*.[6,7]

The absolute requirement for AdoMet led us to propose[6,7] that biotin synthase could belong to the family of enzymes which use AdoMet as a source of desoxyadenosyl radical, such as pyruvate formate lyase,[8] anaerobic ribonucleotide reductase,[9] or lysine 2,3-amino mutase.[10] These three enzymes catalyze homolytic bond cleavage: C–C for pyruvate formate lyase, and C–H for anaerobic ribonucleotide reductase and lysine 2,3-amino mutase. In the first two cases, the desoxyadenosyl radical is relayed by a protein radical, whereas, it is directly responsible for the C–H cleavage in lysine 2,3-amino mutase.

We have recently shown[11] that similar to the case of pyruvate formate lyase[12] and anaerobic ribonucleotide reductase,[13] the natural reducing agent (NADPH) and the electron transport system could be replaced by an artificial electron donor (i.e. photoreduced deazaflavin).

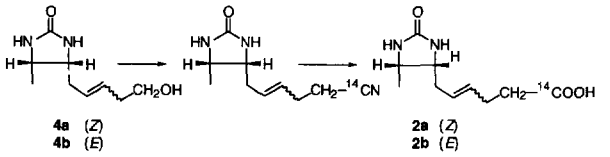


Scheme 2.

Key words: biotin, dehydrodethiobiotin, biotin synthase, labelling.

These new data and the availability of a purified system increased the interest of examining the fate of compounds **2** and **3**.

In this paper we describe the first part of this work, namely, the synthesis of the two isomers of **2** with a ^{14}C-labelled COOH group and our preliminary data concerning their behaviour with the enzymatic in vitro system.



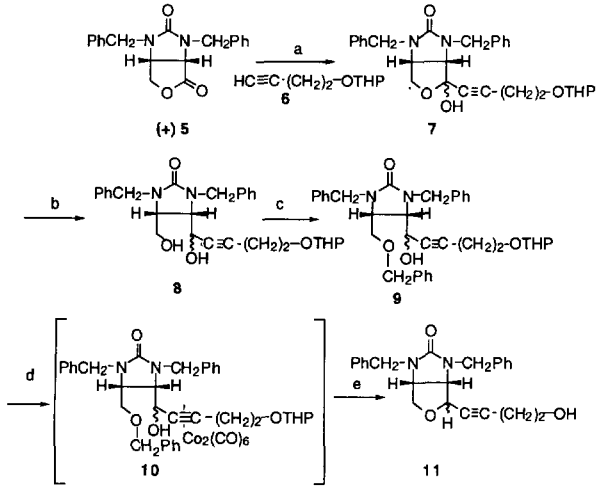
Scheme 3.

Results and Discussion

Chemistry: synthesis of *Z*- and *E*-[1-^{14}C]4,5-dehydrodethiobiotin

Alcohols **4a** (*Z*) and **b** (*E*), which were obvious precursors of the ^{14}C-labelled acids **2a** and **b** (Scheme 3), were our first targets. We considered two possible strategies adapted from synthetic routes to 9-hydroxy dethiobiotin, previously developed in our laboratory.

The first one, described in Scheme 4, used as starting material the lactone (+)**5**, an intermediate in the Hoffmann–La Roche total synthesis of biotin,[14] available in large amounts. The reaction sequence was the same as the one in ref 15 starting with the addition, on lactone **5** of LiC≡C—$(CH_2)_2$OTHP, instead of BrMg$(CH_2)_4$CH=CH_2. The hemiacetal **7** was reduced to **8** with sodium borohydride ($NaBH_4$) and the

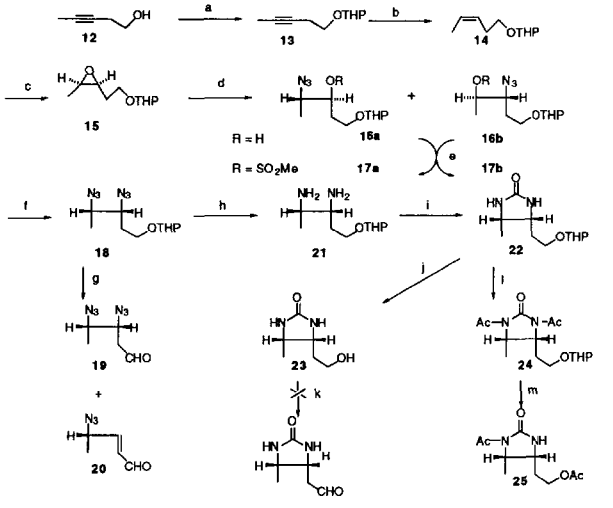


Scheme 4. Reagents and conditions: (a) BuLi, THF, 84% (crude); (b) $NaBH_4$, EtOH, 85%; (c) $PhCH_2Br$, K_2CO_3, $(CH_3)_2CO$, 63%; (d) $Co_2(CO)_8$, CH_2Cl_2; (e) i. $NaBH_4$, TFA, CH_2Cl_2; ii. $Fe(NO_3)_3 \cdot 9H_2O$, CH_2Cl_2, 39%.

primary alcohol protected with a benzyl group. The crucial and risky step was the deoxygenation of the secondary alcohol of **9** because of the possibility of formation of allenic products. This route was, however, attempted since **9** was readily available. However, whereas the deoxygenation was achieved efficiently with the saturated side chain[15] by the Barton–McCombie procedure [i.e. tributyltin hydride (Bu_3SnH) on the corresponding xanthate] it gave only complex mixtures in the case of **9**, and the NMR spectrum of the crude product revealed a high proportion of allenic compounds. We then tried to use the Nicholas method[16] devised for the deoxygenation of propargylic alcohols. In this method the triple bond is protected as a hexacarbonyl dicobalt derivative, which stabilizes a carbonium ion at the α position. Reduction can be achieved with $NaBH_4$ and trifluoroacetic acid (CF_3CO_2H). In our case, participation of the neighbouring oxygen, in spite of its protection, led to the cyclized product, which could be characterized as **11** after removal of the organometallic moiety.

The second strategy involved the construction of the imidazolidinone group, starting from a double bond, namely 9,9-ethylenedioxy-1-tetrahydropyranyloxy-*Z*-non-2-ene.[17] This route was completely stereospecific, leading to the *cis* relationship of the substituents of the imidazolone ring. It was thus transposed to the present case, as depicted in Scheme 5. A Wittig reaction, the most obvious way to obtain the unsaturated side-chain of **4** could be *a priori* carried out at different stages of the synthesis.

The starting material in this route was the pent-3-ynol **12**, which, after protection with dihydropyran and hydrogenation with palladium on barium sulfate ($Pd/BaSO_4$) in the presence of quinoline, gave the *cis*



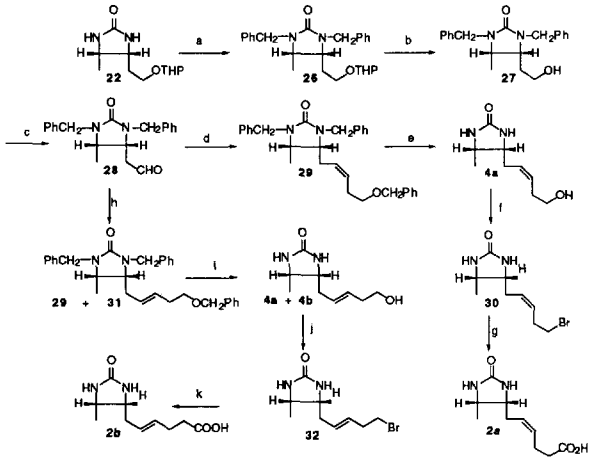
Scheme 5. Reagents and conditions: (a) DHP, PPTs, CH_2Cl_2, 82%; (b) H_2, $Pd/BaSO_4$, quinoline, EtOH, 94%; (c) mCPBA, CH_2Cl_2, 90%; (d) NaN_3, NH_4Cl, EtOH; (e) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 62% from **13**; (f) NaN_3, DMF, 88%; (g) i. PPTs, EtOH, 57%; ii. PCC, CH_2Cl_2 (crude); (h) H_2, $Pd/CaCO_3$/PbO, EtOH, (crude); (i) $(Cl_3CO)_2CO$, NEt_3, CH_2Cl_2, 85% from **18**; (j) PPTs, CH_2Cl_2, 85%; (k) ClCO—COCl, DMSO, CH_2Cl_2, Et_3N, −78 °C; (l) $(CH_3CO)_2O$, NEt_3, (crude); (m) PPTs, EtOH; 28% from **22**.

olefin **14**. The *cis* olefin is always contaminated (3–10%) by the corresponding *trans* isomer. As they are extremely difficult to separate the synthesis was continued on the mixture (see Experimental). Oxidation of **14** with *meta*-chloroperbenzoic acid (*m*CPBA) led to the oxirane **15** which was converted into azido alcohols **16a** and **b** with sodium azide (NaN_3) in ethanol. The corresponding mesylates **17a** and **b** were treated again with NaN_3 in *N,N*-dimethylformamide (DMF) to give the diazide **18**.

The chain elongation was attempted at this stage. After removal of the tetrahydropyranyl (THP) group and oxidation with pyridinium chlorochromate (PCC), the aldehyde **19** was obtained together with 10% of the elimination product **20**. The Wittig reaction with $Ph_3P^+—CH_2—CH_2—CH_2OH$, Br^- did not give the expected product but a complex mixture containing, according to the NMR spectrum, the elongation product of **20**.

Thus the imidazolidinone ring had to be constructed first. Hydrogenation with Lindlar catalyst gave the diamine **21** that was not very stable and was converted without purification into **22**. The cyclization occurred with good yields using triphosgene under high dilution conditions to avoid the formation of polymers. Compound **22** was easily deprotected but unfortunately the oxidation of **23** failed leading to very complex mixtures. Having already experienced the sensitivity of the imidazolidinone ring to oxidizing agents, we decided to protect the nitrogens of **22** first with easy to remove acetyl groups. The diacetate **24** was readily obtained, but all attempts to deprotect the alcohol function led to the transacetylation product **25**.

We had to return to the benzylation of the nitrogens which protects efficiently[15] but was first avoided because of the deprotection conditions. The successful preparation of our target compounds **2a** and **b** is shown in Scheme 6.



Scheme 6. Reagents and conditions: (a) tBuLi, THF/HMPA, −78 °C, $PhCH_2Br$, 50% from **18**; (b) PPTs, EtOH, 70 °C, 70%; (c) ClCO—COCl, DMSO, NEt_3, −78 °C, 94%; (d) $Ph_3P^+—(CH_2)_3—OCH_2Ph$, Br^-, *n*-BuLi, THF/HMPA, 0 °C, 73%; (e) Na, NH_3, EtOH, THF, −78 °C, 96%; (f) Ph_3P, Br_2, pyridine, CH_3CN, 92%; (g) i. KCN, DMSO; ii. NaOH 1 N, 120 °C, 87%; (h) $Ph_3P^+—(CH_2)_3OCH_2Ph$, Br^-, *n*-BuLi, THF, N BuLi, $LiClO_4$, 55% (40/60); (i) cf. (e); (j) cf. (f); (k) cf. (g).

The dilithio derivative of **22** was benzylated and after deprotection and Swern oxidation, the aldehyde **28** was obtained with a good yield. A Wittig reaction with $Ph_3P{=}CH—CH_2—CH_2—OCH_2Ph$ afforded the *cis* olefin which was treated with sodium in liquid ammonia (Na/NH_3), giving the *cis* alcohol **4a**. The coupling constant $^3J_{H7\text{-}H8} = 7.8$ Hz confirmed the expected *cis* relationship between the imidazolidinone substituents.

To obtain **31**, the isomer of **29** with a *trans* double bond, we used the Schlosser modification[18] of the Wittig reaction involving the formation of the betain ylide in the presence of lithium salt. By varying the conditions, we always obtained a mixture of **29** and **31**, the best ratio being 40:60. These two isomers being very difficult to separate, the mixture was debenzylated and **4b** was obtained pure by preparative high-pressure liquid chromatography. Then, the same reaction sequence was applied to both isomers (i.e. transformation of the alcohol into the bromide) treatment with potassium cyanide (KCN), and basic hydrolysis of the nitrile. Using [^{14}C]KCN, we could obtain ^{14}C-labelled **2a** and **b**.

Biochemical experiments

The cyclopropylcarbinyl test that has been widely used as a radical probe in many organic reactions[19] has also been successfully applied to several enzymatic systems,[19–21] although the interpretations are not always straightforward.

The behaviour of ethylenic dehydro analogues of natural substrates has also been examined for instance in the case of isopenicillin N synthase (IPNS)[22] or dopamine β hydroxylase (DBH).[23] Analysis of the transformation products informed about the fate of the intermediates.[22,24] There are also some reports of covalent binding of an intermediate radical to the protein.[23,25]

Before having completed the synthesis of the cyclopropyl or epoxy derivatives **3**, we thought it was of interest to test the ethylenic derivatives **2a** and **b**. Two series of experiments were performed. In the first one, we examined the reaction products of [^{14}C]**2a** and [^{14}C]**2b** to check if they were substrates of biotin synthase and eventually identify new transformation products. The enzymatic preparation used for this experiment was a crude cell-free extract of *B. sphaericus* (CFE)[7] which gave the better conversion yield.

The incubation of [^{14}C]**2a** and [^{14}C]**2b** was carried out with all the necessary cofactors (see Experimental section). After protein precipitation, the supernatant was passed through a Sep-Pak (Waters) cartridge and analyzed by thin-layer chromatography (TLC). Radio-

activity profiles are shown in Figure 1. The *trans* isomer **2b** is partially transformed into several radioactive compounds, whereas no significant transformation is observed in the absence of AdoMet or in the presence of a large excess of the substrate (DTB). This indicates that the reaction occurs in the active site of the enzyme. On the other hand, the extent of the transformation of **2a** was within the detection limit. These very preliminary results, although reproducible, should be substantiated. The in vitro system presently available being not catalytic with the natural substrate, but only sub-stoichiometric with respect to the enzyme, the reaction products of the analogues are formed in very small amounts. A separation, therefore, has not yet been attempted and will be undertaken once the efficiency of the system has been improved.

In the second experiment, we analyzed the protein and for that purpose the reaction was carried out with pure biotin synthase and the simplified in vitro system using photoreduced deazaflavin as electron source,[11] with **2a** or **2b** as substrates. After reaction, the protein was recovered by gel filtration and analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The radioactivity profiles (Fig. 2) were obtained by counting the gel cut into 5 mm pieces.

They clearly show that the protein has been covalently labelled to an extent of ~0.6% with **2a** and of ~1% with **2b**. This labelling is suppressed in the absence of AdoMet or irradiation as well as in the presence of a 20-fold excess of DTB. Although further experiments are necessary, we can postulate that **2a** and **b** are mechanism-based inhibitors of biotin synthase. If we consider that the amount of biotin produced in corresponding experiments carried out with DTB is about 5% with respect to biotin synthase, trapping of the intermediate appears rather efficient. We should easily obtain, *a fortiori* if we improve the turnover of the system, a sufficient amount of the labelled protein for sequencing and identification of the modified amino acid(s). This will provide the first indication on the active site localization. When the same experiment was performed with [^{3}H]dethiobiotin no labelling of the protein was observed. The difference of behaviour between the natural substrate and its unsaturated isomers and between **2a** and **b** probably reflects a different geometry of the enzyme–substrate complex. In the latter cases, a less efficient C–S bond formation would allow the intermediate radical to covalently label the protein.

Conclusion

The synthesis of the two isomers of 4,5-dehydrodethiobiotin **2a** and **b** labelled with ^{14}C has been achieved. These compounds are precursors of the corresponding cyclopropyl and epoxy derivatives, the synthesis of which is in progress. It was also of interest to examine their behaviour with biotin synthase. It was observed that the *trans* isomer [^{14}C]**2b** was transformed by the enzyme into several products which have not yet been identified, whereas, the extent of transformation of the *cis* isomer was within the detection limit. Interestingly, we observed in these experiments a covalent labelling of the protein, probably by trapping of the intermediate radical. This happened with the two isomers, but **2b** was more efficient than **2a**. Thus, both analogues could be the first mechanism-based inhibitors of biotin synthase.

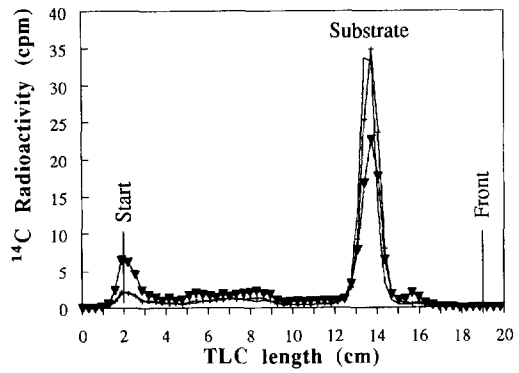


Figure 1. Radioactive TLC profiles of visualization experiments carried out with [^{14}C]**2b**. The standard reaction mixture consisted of CFE (25 mg/mL), 40 μM (±) [^{14}C]**2b**, 1 mM NADPH, 1 mM AdoMet, 0.5 mM cysteine, 5 mM DTT and 40 mM Tris buffer pH 8.0. It was incubated at 37 °C for 1 h (see Experimental): ▼ **2b**; + **2b** without AdoMet; — **2b** + 100-fold excess DTB.

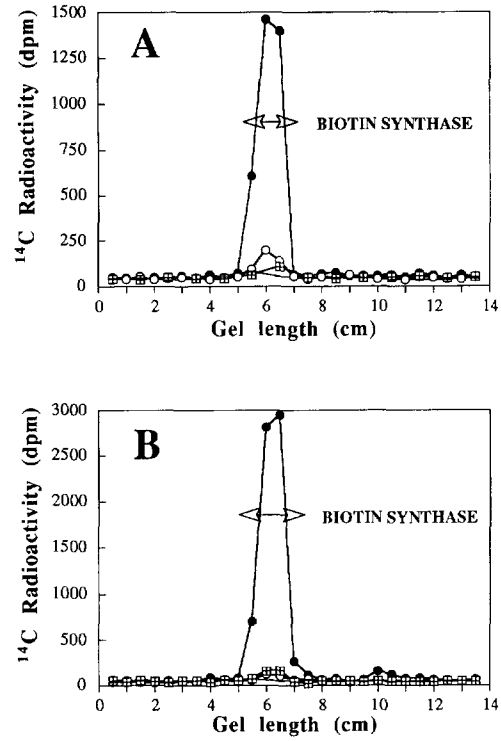


Figure 2. Radioactive electrophoresis gel profiles. The standard labelling assay consisted of pure biotin synthase (2 mg/mL), 200 μM (±) [^{14}C] substrate (**2a** or **b**) and other cofactors as described in the Experimental. Each curve corresponds to 200 μg of protein. (**A**) Substrate [^{14}C]**2a**, (**B**) substrate [^{14}C]**2b**; ● standard labelling assay; ○ standard + 20-fold excess DTB; ⊞⊞ standard without AdoMet; — standard without irradiation.

Sequencing of the labelled protein and identification of the modified amino acid(s) should provide the first characterization of the active site of the enzyme.

Experimental

Chemical synthesis

All reactions using nonaqueous reagents were run under a dry argon atmosphere. Organic layers were dried on $MgSO_4$. Column chromatography was performed on alumina 90 Merck (0.063–0.2 mm) or Kieselgel 60 Merck (70–230 mesh) and flash chromatography on Kieselgel 60 Merck (230–400 mesh). Reactions progress were monitored by analytical TLC on silica gel 60F-254, alumina 150F-254 or neutral alumina F-254 (type E) from Merck. Visualization of TLC was done by phosphomolybdic acid or by UV light. For the amino or amido groups, paradimethylamino cinnamaldehyde was used. ^1H NMR spectra were recorded on a Jeol GSX400 or on a Bruker ARX400. ^{13}C NMR spectra were recorded on a Jeol GSX400 or on a Bruker ARX400 at 100 MHz. Solutions in $CDCl_3$ were used unless otherwise stated, with tetramethylsilane as an internal standard; coupling constants were obtained by spin decoupling. The compounds described in Schemes 5 and 6, were contaminated with a small amount of their stereoisomer at positions 7 and 8. The chemical shift of the methyl group of this minor isomer is given in square brackets. Mass spectral data were obtained by chemical ionization (CI NH_3) or by electron impact (EI) on a Nermag R10–10C or R30–10 spectrometer. Infrared spectral data were recorded on a Perkin 1420 (values in cm^{-1}). UV-Vis measurements were made on an Uvikon 390 spectrophotometer. Elemental analyses were performed by the Service Régional de Microanalyse (SIAR-Jussieu). All chemicals were purchased from Aldrich, Janssen and Sigma. [^{14}C]KCN was from NEN. Analytical HPLC was performed with Waters pumps, a 759 A absorbance detector (Applied Biosystems), a Merck D 2500 chromato integrator and a Kromasil RP C8 5 μ column (4.6 × 250 mm). Preparative HPLC was performed on a 400 solvent delivery system, a 1480 A injector, a 783 A programmable absorbance detector (Applied Biosystems) and an Aquapore octyl 20 μ Brownlee column (10 × 250 mm). Counting was performed with an LKB 1214 Rackbeta scintillation counter, using Aquasol-2 (Packard) as scintillation liquid if not specified. Radioactive TLC plates were scanned with an automatic TLC linear analyzer Berthold LB 2821.

1-(Tetrahydropyranyl-2-oxy)but-3-yne (6). Dihydropyran (9 g, 107 mmol) and 1 g of pyridinium *p*-toluenesulfonate (PPTs) were added to 10 g (142.8 mmol) of 1-hydroxy but-3-yne dissolved in 80 mL of CH_2Cl_2. After stirring for 3 h at room temperature, the soln was washed with an $NaHCO_3$ soln, then H_2O and dried. Distillation (bp 81 °C, 19 mm) gave 18.9 g (86%) of **6**: IR ($CHCl_3$) 3300 (H—C≡C), 2150 (C≡C); ^1H NMR: δ 1.55–1.85 (m, 6H, 3-CH_2-), 1.97–1.98 (br s, 1H, H—C≡-), 2.50–2.51 (m, 2H, -≡-CH_2-), 3.51–3.61, 3.82–3.91 (2m, 2 × 2H, 2-CH_2O-), 4.65–4.66 (m, 1H, -O—CH—O-); ^{13}C NMR: δ 19.17; 19.74; 25.23; 30.33 (-CH_2-); 61.93; 65.29 (-CH_2O-); 69.06 (≡C-); 81.19 (HC≡); 98.51 (-O—CH—O-). Anal. calcd for $C_9H_{14}O_2$: C, 70.16; H, 9.09. Found: C, 70.07; H, 9.24.

***cis*-1,3-Dibenzyl-4-hydroxy-4-[4-(tetrahydropyranyl-2-oxy)-but-1-ynyl]-2,3,3a,4,6,6a-hexahydrofuroimidazol-2-one (7).** *n*-Butyllithium (1.6 M in hexane) (4.26 mL, 6.8 mmol) was added dropwise at 0 °C to a solution of 1 g of **6** (6.5 mmol) in 60 mL of anhydrous THF. The mixture was stirred for 1 h at 0 °C then lactone **5** (2.1 g, 6.5 mmol) in 30 mL anhydrous THF was added. After further stirring for 1 h at 0 °C, a satd NH_4Cl soln was added to the mixture, THF was evapd under vacuum, and the product extracted with CH_2Cl_2. The organic layers were washed with H_2O, dried, and evapd. Purification performed on a flash silica gel column ($CHCl_3$:CH_3OH, 95:5) gave 2.62 g (84%) of **7** as an oil: ^1H NMR: δ 1.35–1.80 (m, 6H, 3-CH_2-), 2.54 (2.43) (m, 2H, -CH_2-≡), 3.40–3.49, 3.53–3.62, 3.66–3.86 (m, 8H, 3-CH_2-O, 2-CHN-), 4.07 [4.01] (d, 1H, J = 15.4 Hz [14.8], -NCHHPh), 4.39 [4.41] (d, 1H, J = 15.4 Hz [14.8], -NCHHPh), 4.56–4.59 [4.50–4.54] (m, 1H, -O—CH—O-), 4.71 [4.63] (d, 1H, J = 15.4 Hz [14.8], -NCHHPh), 5.05 [4.98, 5.06] (d, 1H, J = 15.4 Hz [14.8], -NCHHPh), 7.21–7.35 (m, 10H, arom.); ^{13}C NMR: δ 19.08 [19.19] (-CH_2-≡), [19.76] 19.98, 25.08 [25.36]; 30.26 (-CH_2-), 46.07 [46.33], [46.44] 46.66 (-NCH_2Ph), 56.44, 57.49 (-CHN-), 62.04 [61.95, 62.17], 64.86 [64.12, 65.04, 65.29], 68.16 [67.66, 68.66] (-CH_2O-), 77.33 [77.58], 84.55 [83.14, 84.62] (-C≡C-), 98.52, 99.48 [97.74, 98.47, 98.58, 98.61, 98.65] (-C(OH)- and -O—CH—O-), 127.26, 127.35 [127.50], 127.84 [128.02], 128.40, 128.46, 136.47, 136.62 (arom.), 159.17 [159.83] (C=O).

***cis*-1,3-Dibenzyl-5-hydroxymethyl-4-[1-hydroxy-5-(tetrahydropyranyl-2-oxy)pent-2-ynyl]-imidazolidin-2-one-(8).** Sodium borohydride (84 mg, 2.2 mmol) was added slowly to a soln of 210 mg (0.44 mmol) of **7** in 5 mL of absolute ethanol at −50 °C. The mixture was stirred for 5 h and poured at 0 °C in 2 mL of satd aq NH_4Cl. After stirring for 15 min, EtOH was evapd under vacuum, the compound was extracted with $CHCl_3$, and the organic layer washed with H_2O to pH 7. After drying, evapn in vacuo, purification by silica gel flash chromatography ($CHCl_3$:CH_3OH, 95:5) 179 mg (85%) of **8** were obtained as an oil: ^1H NMR: δ 1.36–1.73 (m, 6H, 3-CH_2-), 1.76 (s, 1H, OH), 2.40–2.44 (m, 2H, -CH_2-≡), 3.43–3.51, 3.59–3.81 (2m, 7H, 2-CH_2O-, 2-CHN-, -CHHOH), 3.98–4.12 (m, 2H, -CHH-OH, -NCHHPh), 4.27–4.36 (d, 1H, -NCHHPh), 4.48–4.55 (m, 1H, -O—CH—O-), 4.66–4.71 (m, 1H, -CHOH), 4.71–4.77 (m, 1H, -NCHHPh), 4.93–5.01 (d, 1H, J = 16 Hz, -NCHHPh), 7.19–7.26 (m, 10H, arom.); ^{13}C NMR: δ 19.15 (-CH_2-), 19.92 (-CH_2-≡); 24.97, 30.16 (-CH_2-), 45.54, 45.87 (-N-CH_2Ph), 56.75 (-CHN-), 57.47 (-CH_2OH), 58.03 (-CHN-), 59.68 (-CHOH), 62.11, 65.09 (-CH_2O-), 79.63, 83.78 (-C≡C-), 98.60

(-O—CH—O-), 126.98, 127.07, 127.44, 127.55, 128.15, 128.26, 136.80, 136.91 (arom.), 161.31 (C=O).

***cis*-1,3-Dibenzyl-5-benzyloxymethyl-4-[1-hydroxy-5-(tetrahydropyranyl-2-oxy)pent-3-ynyl]-imidazolidin-2-one (9).** Benzyl bromide (27.3 μL, 0.23 mmol) was added to 100 mg (0.21 mmol) of **6** and 17.2 mg (0.23 mmol) of K_2CO_3 in 2 mL of acetone. After refluxing with stirring overnight and evapn under vacuum, the product was extracted with $CHCl_3$, the organic layer was washed with H_2O, dried, and after evapn in vacuo, purified by silica gel flash chromatography ($CHCl_3$: CH_3OH, 95:5) giving 75 mg (63%) of **9** as an oil: MS EI ($M^{+\cdot}$)=568; ^1H NMR: δ 1.40–1.80 (m, 6H, 3-CH_2-), 2.46 (dt, 2H, J=1.6 and 6.6 Hz, -CH_2-≡), 3.41–3.83 (m, 7H, 2-CH_2O-, 2-CHN-, -CHH-O-CH_2Ph), 3.96–4.04 (m, 2H, -CHH-O-CH_2Ph, -NCHHPh), 4.23 (d, 1H, J=15.4 Hz, -NCHHPh), 4.26–4.36 (m, 2H, -CHOH, -OCHHPh), 4.52–4.60 (m, 2H, -NCHHPh, -O—CH—O-), 4.72 (d, 1H, J=11.5 Hz, -OCHHPh), 4.85 (d, 1H, J=15.4 Hz, -NCHHPh), 7.00–7.38 (m, 15H, arom.); ^{13}C NMR: δ 19.14 [19.19] (-CH_2-), 20.12 (-CH_2-≡), 25.08, 30.26 (-CH_2-), 46.06, 46.28 (-NCH_2Ph), 57.19, 57.56 (-CHN-), 58.49 (-CH_2-O-CH_2Ph), 61.99 [62.08], 65.11 (-CH_2O-), 66.50 (-CHOH), 70.60 (-OCH_2Ph), 76.18, 87.00 (-C≡C-), 98.58 [98.65] (-O—CH—O-), 126.95, 127.00, 127.51, 127.75, 127.88, 127.99, 128.17, 128.26, 136.23, 137.00, 137.50 (Arom.), 161.18 (C=O).

***cis*-1,3-Dibenzyl-4-(4-hydroxybut-1-ynyl)-2,3,3a,4a,6,6a-hexahydrofuroimidazol-2-one (11).** Octacarbonyldicobalt (164 mg, 0.48 mmol) was added under argon to a soln of **9**, 272 mg (0. 48 mmol) in 5 mL of anhydrous CH_2Cl_2. After stirring for 5 h at room temperature, the solvent was evapd under vacuum. The NMR spectrum of the crude product was consistent with structure **10**: ^{13}C NMR: δ 19.61 [19.66], 25.22 [25.35], 30.31 [30.40] (-CH_2-), 33.64 [33.69] (-CH_2-≡), 46.39, 48.83 (-NCH_2Ph), 55.62 [55.80] (-CHN-), 58.67 [58.78] (-CHN-), 59.79 [59.95] (-CH_2OCH_2Ph), 62.68 [62.79], 67.42 [67.51] (-CH_2O-), 74.46 [74.55] (-CHOH), 78.97 (-OCH_2Ph), 94.21 [94.39], 95.82 [96.04] (-C≡C-), 98.98 [99.20] (-O—CH—O-), 127.22, 127.62, 127.68, 127.73, 127.97, 128.04, 128.17, 128.32, 128.48, 128.51, 128.59, 128.72, 128.81, 136.69, 136.95, 136.98, 137.26 (arom.), 162.61 (-N—C(O)—N-), 199.35, 199.64 (C=O).

To a soln of this crude product in 5 mL of CH_3OH, $NaBH_4$ (54 mg, 1.4 mmol) was added at 0 °C and then 500 μL of CF_3COOH. After stirring for 3 h, the mixture was poured in cold water (50 mL) and the product extracted with CH_2Cl_2 (100 mL). The organic layer was washed with H_2O to pH 7, dried and concd in vacuo. $Fe(NO_3)_3 \cdot 9H_2O$ (775 mg, 1.92 mmol) was added to the crude product, dissolved in CH_2Cl_2 (5 mL). After stirring 2 h at room temperature, 100 mL of CH_2Cl_2 were added, the organic layer was washed with H_2O (2×20 mL), dried, and evapd under vacuum. Purification by silica gel flash chromatography (C_6H_{12}:$CH_3COOC_2H_5$, 1:1) gave 70 mg (39%) of **11** as an oil. ^1H NMR: δ 1.62 (s, 1H, -OH), 2.29–2.33 (m, 2H, -CH_2-≡), 3.56 (t, 2H, J=6.3 Hz, -CH_2OH), 3.67–3.70 (dd, 1H, J=10, 4 Hz, -CHH-O-), 3.76 (d, J=10 Hz, -CHH-O-), 3.87–3.93 (m, 2H, 2-CHN-), 4.10, 4.21, 4.63, 4.69 (4d, 4H, J=15.2 Hz, -NCH_2Ph), 4.44 (s, 1H, -O-CH-≡), 7.10–7.35 (m, 10H, arom.); ^{13}C NMR: δ 22.87 (-CH_2-≡), 46.48, 46.97 (-NCH_2Ph), 57.65 (-CHN-), 60.61 (-CH_2OH), 64.98 (-CHN-), 69.89 (-CH_2O-), 72.68 (O-CH-≡), 77.84, 84.86 (-C≡C-), 127.64, 128.08, 128.13, 128.70, 136.62, 136.69 (arom.), 159.01 (C=O). Anal. calcd for $C_{23}H_{24}N_2O_3$: C, 73.43; H, 6.38; N: 7.44. Found: C, 73.05; H, 6.30; N, 7.35.

1-(Tetrahydropyranyl-2-oxy)pent-3-yne (13). Dihydropyran 7.72 g (91.8 mmol) and PPTs (1 g) were added to a soln of pent-3-ynol **12** (5.1 g, 60.7 mmol) in CH_2Cl_2 (60 mL). After stirring for 3 h, the organic phase was washed with Na_2CO_3 (10%), then with H_2O. After drying, evapn and distillation (bp 118 °C 18 mm) 8.36 g (82%) of **13** were obtained: ^1H NMR: δ 1.54–1.85 (m, 9H, -CH_3, 3-CH_2-), 2.42–2.44 (m, 2H, -CH_2-≡), 3.46–3.58, 3.74–3.94 (2m, 2×2H, 2-CH_2O-), 4.65–4.69 (m, 1H, -O—CH—O-); ^{13}C NMR: δ 2.88 (-CH_3), 19.00 (-CH_2-), 19.67 [19.72] (-CH_2-≡), 25.10, 30.16 (-CH_2-), 61.53, 65.70 (-CH_2-O), 75.44, 75.90, (-C≡C-), 98.17 [98.58] (-O-CH-O-). Anal. calcd for $C_{10}H_{16}O_2$: C, 71.43; H, 9.52; N: 7.44. Found: C, 71.30; H, 9.63.

***Z*-1-(Tetrahydropyranyl-2-oxy)pent-3-ene (14).** $Pd/BaSO_4$ 5% (1 g) and quinoline (1.12 mL) were added to 30 mL of ethanol 95 °C. The mixture was stirred for 30 min under vacuum and 60 min under H_2. Compound **13** (8 g, 49.7 mmol) was added and after stirring until 1.12 L of H_2 was absorbed, the catalyst was washed with EtOH 95 °C. Ethanol was evapd and the residue dissolved in Et_2O, the organic layer was washed with 0.1 N HCl, then with Na_2CO_3 (10%), dried, and evapd under vacuum yielding crude **14** (7.94 g): ^1H NMR: δ 1.54–1.92 (m, 9H, -CH_3, 3-CH_2-), 2.39–2.44 (m, 2H, -CH_2-=), 3.44–3.59, 3.75–3.96 (2m, 2×2H, 2-CH_2O-), 4.65–4.69 (m, 1H, -O—CH—O-), 5.44–5.55 (m, 1H, -CH=), 5.56–5.63 (m, 1H, =CH-); ^{13}C NMR: δ 12.24 [12.33] (-CH_3), 19.02, 25.13 [25.19], 27.16 [27.23], 30.25 (-CH_2-), 61.43, 66.32 [66.37] (-CH_2O-), 97.98 [98.12] (-O—CH—O-), 125.08 (-CH=), 126.14 (=CH-).

***cis*-2-[2-(Tetrahydropyranyl-2-oxy)ethyl]3-methyloxirane (15).** Compound **14** (5 g, 29.4 mmol) was dissolved in CH_2Cl_2 (30 mL). At −78 °C *m*CPBA (46% in H_2O) (12.13 g, 32.3 mmol) in CH_2Cl_2 (60 mL) was added dropwise. The mixture was stirred at room temperature overnight, then washed with 0.5 N NaOH and H_2O, dried, and evapd under vacuum. The crude product was purified on basic alumina (C_6H_{12}:$CH_3COOC_2H_5$, 95:5) yielding 4.92 g (90%) of **15**: ^1H NMR: δ 1.3 (m, 3H, -CH_3), 1.4–2.0 (m, 8H, 4-CH_2-), 3.0–3.2 (m, 2H, -CH oxirane), 3.5–3.7, 3.8–4.1 (2m, 2×2H, 2-CH_2O-), 4.6 (m, 1H, -O—CH—O-); ^{13}C NMR: δ 13.28 [13.34] (-CH_3), 19.39 [19.52], 25.41, 28.21 [28.57], 30.58 (-CH_2-); 52.49, 54.71 (-CH-oxirane), 62.07 [62.29], 64.42 [64.60] (-CH_2O-),

98.67 [98.91, 99.03] (-O—CH—O-). Anal. calcd for $C_{10}H_{18}O_3$: C, 64.51; H, 9.68. Found: C, 64.74; H, 9.98.

2-Azido-5-(tetrahydropyranyl-2-oxy)pentane-3-ol (16a). 3-Azido-5-(tetrahydropyranyl-2-oxy)-pentane-2-ol (16b). Sodium azide (2.34 g, 36 mmol) and $(NH_4)Cl$ (1.93 g, 36 mmol) were added to a soln of **15** (5.15 g, 27.6 mmol) in 55 mL of EtOH 80%. The mixture was refluxed for 8 h. Then H_2O was added, ethanol was evapd and the product extracted with Et_2O. The organic layers were washed with H_2O, dried, and evapd under vacuum. A mixture of **16a** and **b** was obtained: IR: 2100 (N_3), 3430 (-OH); ^{1}H NMR: δ 1.27–1.34 (m, 3H, -CH_3), 1.53–1.85 (m, 9H, 4-CH_2-, -OH), 3.40–4.03 (m, 6H, 2-CH_2O-, -CH-OH, -CHN_3), 4.57–4.63 (m, 1H, -O—CH—O-); ^{13}C NMR: δ 15.38, 15.44 (-CH_3), 19.45, 19.67, 19.80, 20.02, 20.07, 25.21, 25.32, 30.42, 30.53, 30.60, 30.68, 31.07, 31.12, 33.05 (-CH_2-), 61.62 (-CH-N_3), 62.30, 62.37, 62.66, 62.70, 63.80, 64.13, 65.33, 65.59 (-CH_2O-), 65.70, 65.74 (-CHN_3), 69.65, 69.85, 73.25, 73.93 (-CHOH), 98.78, 99.10, 99.39, 99.48 (-O—CH—O-).

4-Azido-3-mesyloxy-1-(tetrahydropyranyl-2-oxy) pentane (17a). 3-Azido-4-mesyloxy-1-(tetrahydropyranyl-2-oxy) pentane (17b). At 0 °C the crude mixture of **16a** and **b** (2 g, 8.7 mmol) was dissolved in pyridine (10 mL) and methane sulphonyl chloride (845 μL, 10.9 mmol) was added. The mixture was stirred for 3 h at 0 °C and 1 h at room temperature, then poured into H_2O (10 mL). The product was extracted with $CHCl_3$. The organic layers were washed with H_2O, dried, and evapd under vacuum. Purification on basic alumina (C_6H_{12}:$CH_3COOC_2H_5$, 8:2) yielded a mixture of **17a** and **b** (1.3 g, 62% from **13**); IR: 2100 (N_3); ^{1}H NMR: δ 1.39 (m, 3H, -CH_3), 1.50–2.1 (m, 8H, 4-CH_2-), 3.01–3.12 (s, 3H, CH_3SO_2-), 3.50–3.95 (m, 5H, 2-CH_2O-, -CH-N_3), 4.56–4.61 (m, 1H, -O-CH-O-), 4.72–4.84 (m, 1H, -CH-OMs); ^{13}C NMR: δ 15.07 [15.26], 18.44 (-CH_3), 19.47 [19.59, 19.72], 25.26, 30.51 [30.60, 30.64], 31.04, 31.48 (-CH_2-), 38.39 [38.43], 38.58 (CH_3SO_2-), 58.64 [58.69] (-CH—N_3), 62.59 [62.70, 62.77], 63.10 [63.32] (-CH—N_3—CH_2—O), 80.24 [80.35], 81.19 [81.66] (-CHOMs), [98.78] 99.09, 99.42 (-O—CH—O-).

3,4-Diazido-1-(tetrahydropyranyl-2-oxy)pentane (18). Under argon, sodium azide (5.4 g, 83.4 mmol) was added to a soln of **17a** and **b** (12.8 g, 41.7 mmol) in DMF (85 mL). The mixture was stirred at 100 °C for 24 h then H_2O (100 mL) was added and the product extracted with Et_2O. The organic layers were washed with H_2O, dried, and evapd under vacuum. Purification on basic alumina (C_6H_{12}:$CH_3COOC_2H_5$, 95:5) yielded 9.36 g (88%) of **18**; IR: 2100 (N_3); ^{1}H NMR: δ 1.30–1.33 (m, 3H, -CH_3), 1.53–1.92 (m, 8H, 4-CH_2-), 3.47–3.94 (m, 6H, 2-CH_2O-, 2-CHN_3), 4.58–4.61 (m, 1H, -O-CH-O-); ^{13}C NMR: δ 14.38, 14.42 (-CH_3), 19.41, 19.63, 25.32, 30.55, 30.59, 30.91 (-CH_2-), 60.25, 60.40 (-CHN_3), 62.26, 62.59 (-CH_2O-), 63.30, 63.41 (-CHN_3), 63.50, 63.74 (-CH_2O-), 98.65, 99.40 (-O—CH—O-).

3,4-Diazido pentanal (19). 4-Azido pent-2-enal (20). Diazide **18** (1 g, 3.9 mmol) and PPTs (109 mg, 0.39 mmol) in EtOH (60 mL) were stirred at 60 °C for 6 h, EtOH was then evapd under vacuum. After flash chromatography on silica gel ($CHCl_3$:CH_3OH, 95:5), the alcohol was obtained (380 mg, 57%) as a colorless oil; ^{1}H NMR: δ 1.33 (d, 3H, J=6.6 Hz, -CH_3), 1.62–1.71 (m, 2H, -CHH-, -OH), 1.78–1.86 (m, 1H, -CHH-), 3.60–3.65 (m, 2H, 2-CHN_3), 3.79–3.85 (m, 2H, -CH_2OH); ^{13}C NMR: δ 14.36 (-CH_3), 32.83 (-CH_2-), 58.80 (-CH_2O-), 60.23–62.86 (-CHN_3).

PCC (1 g, 4.64 mmol) was added to this crude product (500 mg, 2.94 mmol) in anhydrous CH_2Cl_2 (20 mL). After stirring at room temperature for 3.5 h, the solvent was evapd under vacuum and Et_2O (20 mL) was added to the residue. After filtration and washing of the precipitate with Et_2O, the organic phase was dried and evapd. The ^{1}H NMR spectrum of the crude product revealed the presence of two compounds **19** (90%) and **20** (10%).

19: ^{1}H NMR: δ 1.31 (d, 3H, J=6.6 Hz, -CH_3); 2.65–2.71 (m, 2H, -CH_2-), 3.42–3.69 (m, 2H,-CHN_3), 9.81 (s, 1H, -CHO).

20: ^{1}H NMR: δ 1.44 (d, 3H, J=6.6 Hz, -CH_3), 4.23–4.41 (m, 1H, -CHN_3), 6.24–6.30 (m, 1H, -CH=), 6.55–6.71 (m, 1H, =CH-), 9.6 (d, 1H, J=7.7 Hz, -CHO); ^{13}C NMR: δ 19.06 (-CH_3), 57.74 (-CHN_3), 132.25 (-CH=), 153.47 (=CH-), 192.86 (-CHO).

3,4-Diamino-1-(tetrahydropyranyl-2-oxy)pentane (21). A soln of **18** (1 g, 3.9 mmol) in Et_2O (50 mL) was stirred under hydrogen overnight in the presence of Pd/$CaCO_3$, PbO 5% (0.4 g). After filtration, the catalyst was washed with EtOH. Solvent evapn gave the crude diamine **21** (725 mg) as an oil; IR: 3345 (NH_2), MS CI (MH^+)=203; ^{1}H NMR: δ 1.04 [1.08] (d, 3H, J=6 Hz, -CH_3), 1.47–1.84 (m, 12H, 4-CH_2-, 2-NH_2), 2.77–2.93 (m, 2H, 2-CHN-), 3.49–3.58, 3.83–3.95 (m, 4H, 2-CH_2O-), 4.58–4.61 (m, 1H, -O-CH-O-); ^{13}C NMR: δ 17.49–17.64 (-CH_3), 19.50, 25.21, 30.48, 30.55, 32.98, 33.07 (-CH_2-), 51.07, 54.21, 54.56 (-CHN-), 62.24, 62.30, 65.24, 65.64 (-CH_2O-), 98.65, 98.91 (-O—CH—O-).

***cis*-5-Methyl-4-[2-(tetrahydropyranyl-2-oxy)ethyl] imidazolidin-2-one (22).** Triphosgene (0.465 g, 0.17 mmol) was added at 0 °C to a soln of **21** (100 mg, 0.49 mmol) and triethylamine (100 mg, 0.99 mmol) in CH_2Cl_2 (60 mL). The mixture was stirred 1 h at 0 °C and overnight at room temperature, then H_2O was added and the organic phase washed with 0.5 N HCl. After drying, evapn, and alumina chromatography ($CHCl_3$:CH_3OH, 99:1) 96 mg (85%) of **22** was obtained; ^{1}H NMR: δ 1.16 [1.25] (d, 3H, J=6 Hz, -CH_3), 1.47–1.87 (m, 8H, 4-CH_2-), 3.47–3.59 (m, 2H, 2-CHN-), 3.79–3.93 (m, 4H, 2-CH_2O-), 4.55–4.58 (m,

1H, -O CH O-); ^{13}C NMR: δ 15.64 (-CH_3), 19.39, 19.45, 25.06, 29.38, 30.29, 30.37 (-CH_2-), 51.18, 54.27, 54.58 (-CHN-), 62.17, 62.24, 65.11, 65.33 (-CH_2O-), 98.69, 98.71 (-O—CH—O-), 163.69, 163.74 (C=O). Anal. calcd for $C_{11}H_{20}N_2O_3$: C, 57.89; H, 8.77. Found: C, 55.82; H, 8.30.

***cis*-4-(2-Hydroxyethyl) 5-methyl imidazolidin-2-one (23).** A solution of **22** (112 mg, 0.49 mmol) and PPTs (11.2 mg, 0.04 mmol) in EtOH (6 mL) was stirred for 6 h in an oil bath at 100 °C. The solvent was evapd and after flash silica gel chromatography ($CHCl_3$:CH_3OH, 8:2) 60 mg (85%) of **22** was obtained as a white powder; mp 126 °C; IR: 1700 (C=O); ^{1}H NMR: δ 1.02 (d, 3H, J=6 Hz, -CH_3), 1.59–1.64 (dt, 2H, J=1.6, 5 Hz, -CH_2-), 3.54–3.58 (m, 2H, -CH_2O-), 3.73–3.77 (m, 2H, 2-CHN-); ^{13}C NMR (100 MHz): δ 15.84 (-CH_3), 33.40 (-CH_2-), 52.71, 55.07 (-CHN-), 60.50 (-CH_2O-), 166.01 (C=O). Anal. calcd for $C_6H_{12}N_2O_2$: C, 48.64; H, 8.10. Found: C, 48.53; H, 7.79.

***cis*-1,3-Diacetamido-5-methyl-4-[2-(tetrahydropyranyl-2-oxy)ethyl] imidazolidin-2-one (24).** Compound **22** (120 mg, 0.53 mmol) was added to a soln of triethylamine (1.5 mL, 10.5 mmol) and acetic anhydride (4.5 mL, 48 mmol). After stirring for 3 h at room temperature, acetic anhydride was evapd under vacuum and CH_2Cl_2 was added to the residue. CH_2Cl_2 was then washed with 0.1 N NaOH and with H_2O to pH 7, dried, and evapd under vacuum; 180 mg of **24** was obtained as a crude oil. ^{1}H NMR: δ 1.39 (d, 3H, J=6 Hz, -CH_3), 1.54–2.40 (m, 8H, 4-CH_2-), 2.53–2.55 (2s, 2×3H, CH_3CO), 3.40–3.56, 3.80–3.90 (2m, 2×2H, 2-CH_2O-), 4.27–4.36 (m, 2H, 2-CHN-), 4.52–4.58 (m, 1H, -O—CH—O-).

***cis*-1-Acetamido-4-(2-acetoxy ethyl) 5-methyl imidazolidin-2-one (25).** Compound **24** (60 mg, 0.17 mmol) and PPTs (5 mg, 0.018 mmol) in EtOH (6 mL) were stirred for 6 h in an oil bath at 100 °C. The solvent was evapd and after silica gel flash chromatography ($CHCl_3$:CH_3OH, 99:1) 12.7 mg (28%) of **25** was obtained; ^{1}H NMR: δ 1.17 (d, 3H, J=6 Hz, -CH_3), 1.79–1.81 (m, 2H, -CH_2-), 2.03, 2.41 (2s, 2×3H, 2-CH_3CO), 3.71–3.81 (m, 1H, -CHN-), 3.92–4.07 (m, 1H, -CHH-O-), 4.26–4.31 (m, 1H, -CHH-O-), 4.41–4.48 (m, 1H, -CHN-).

***cis*-1,3-Dibenzyl-5-methyl-4-[2-(tetrahydropyranyl-2-oxy)ethyl] imidazolidin-2-one (26).** *tert*-Butyllithium 1.5 M in pentane (13.3 mL, 19.9 mmol) was added dropwise at −78 °C under argon to a soln of **22** (2.27 g, 10 mmol) in an anhydrous mixture of THF:hexamethylphosphoramide (HMPA) 9:1. After stirring for 10 min, benzyl bromide (3.56 mL, 29.9 mmol) was added. The soln was stirred overnight at room temperature. After extraction with CH_2Cl_2, drying, evapn and flash silica gel chromatography (C_6H_{12}:$CH_3CO_2C_2H_5$, 1:1) 2.4 g (50% from **21**) of **26** was obtained; ^{1}H NMR: δ [1.03] 1.06 (d, 3H, J=6 Hz, -CH_3), 1.39–1.91 (m, 6H, 3-CH_2-), 3.22–3.30, 3.35–3.46, 3.59–3.69 (3m, 6H, 2-CH_2O-, 2-CHN-), 3.97 [3.98], 4.15 [4.17], 4.73 [4.76], 4.88 [4.89] (4d, 4H, J=15.4 Hz, -NCH_2Ph), 4.38–4.43 (m, 1H, -O—CH—O-), 7.22–7.32 (m, 10H, arom.); ^{13}C NMR: δ 11.89, 11.96 (-CH_3), 19.35 [19.39], 25.26, 27.16 [27.31], 30.40 [30.46] (-CH_2-), 45.03, 45.89 (-NCH_2Ph), 51.60, 54.74 [55.05] (-CHN-), 62.17 [62.24], 63.94 [64.27] (-CH_2O-), 98.58 [98.89] (-O—CH—O-), 127.14, 127.20, 127.91, 127.99, 128.46, 137.60 [137.64], 137.71 [137.77] (arom.), 160.89 [160.92] (C=O). Anal calcd. for $C_{25}H_{32}N_2O_3$: C, 73.55; H, 7.83; N, 6.85. Found: C, 73.45; H, 7.87; N, 7.01.

***cis*-1,3-Dibenzyl-4-(2-hydroxyethyl)-5-methyl-imidazolidin-2-one (27).** A soln of **26** (2.45 g, 6 mmol) and PPTs (0.151 g, 0.6 mmol) in EtOH (80 mL) was stirred at 60 °C for 4 h, then the solvent was evapd and the crude product purified by flash chromatography on silica gel (C_6H_{12}:$CH_3CO_2C_2H_5$, 1:1). 1.36 g (70%) of **27** was obtained as an oil; ^{1}H NMR: δ [0.98] 0.99 (d, 3H, J=5.5 Hz, -CH_3), 1.59–1.79 (m, 2H, -CH_2-), 3.35–3.44, 3.45–3.50 (2m, 4H, 2-CHN-; -CH_2O-), 3.92, 4.11, 4.67, 4.82 (4d, 4H, J=15.4 Hz, 2-NCH_2Ph), 7.17–7.27 (m, 10H, arom.); ^{13}C NMR: δ 11.73 (-CH_3), 29.89 (-CH_2-), 44.80, 45.69 (N-CH_2Ph), 51.51, 54.52 (-CHN), 58.82 (-CH_2-O), 127.02, 127.13, 127.51, 127.64, 128.28, 128.33, 137.22, 137.52 (arom.), 160.91 (C=O). Anal calcd for $C_{20}H_{24}N_2O_2$: C, 74.09; H, 7.40; N, 8.63. Found: C, 74.02; H, 7.40; N, 8.57.

***cis*-1,3-Dibenzyl-5-methyl-4-(oxyethyl) imidazolidin-2-one (28).** Oxalyl chloride (584 μL, 6.67 mmol) was added dropwise to dimethylsulfoxide (DMSO) (943 μL, 13.3 mmol) dissolved in 15 mL of anhydrous CH_2Cl_2 at −78 °C, under argon. After 10 min stirring, **27** (1.8 g, 5.56 mmol) dissolved in 5 mL of CH_2Cl_2 was added slowly. The solution was stirred for 30 min then triethylamine (2.32 mL, 16.7 mmol) was added and the mixture further stirred at −78 °C for 40 min. After addition of water, the product was extracted with CH_2Cl_2. The organic phase was washed with a satd NaCl soln, dried, and evapd. Silica gel chromatography (C_6H_{12}:$CH_3CO_2C_2H_5$, 1:1) gave 1.69 g (94%) of **28** as an oil: ^{1}H NMR: δ 0.91 [1.06] (d, 3H, J=6.5 Hz, -CH_3), 2.56–2.59 (m, 2H, -CH_2-), 3.52–3.59 (dq, 1H, J=6.5, 7.8 Hz, -CHN-), 3.79–3.84 (m, 1H, J=7.8 Hz, -CHN-), 3.96, 4.14, 4.62, 4.82 (4d, 4H, J=16 Hz, 2-NCH_2Ph), 7.18–7.28 (m, 10H, arom.); ^{13}C NMR: δ 12.68 (-CH_3), 42.23 (-CH_2-), 45.14, 45.20 (-NCH_2Ph), 51.08, 52.20 (-CHN-), 127.42, 127.49, 127.75, 127.97, 128.59, 128.68 (arom.), 160.39 (C=O), 199.45 (-CHO); MS EI, 70 eV ($M^{+\cdot}$): 322; Anal. calcd For $C_{20}H_{22}N_2O_2$: C, 74.55; H, 6.83; N, 8.69. Found: C, 74.41; H, 7.04; N, 8.56.

***cis*-1,3-Dibenzyl-4-(*Z*-5-benzyloxy pent-2-enyl)-5-methyl-imidazolidin-2-one (29).** *n*-Butyllithium (1.6 N) in hexane (1.6 mL, 2.46 mmol) was added at 0 °C to a soln of the phosphonium salt[26] (1.3 g, 2.65 mmol) in 6.3 mL THF and 0.7 mL HMPA. Aldehyde **28** (660 mg,

2.04 mmol) dissolved in 3 mL THF was then added. After 10 min stirring, water was added. The Et_2O extract was dried and evapd. After silica gel chromatography (C_6H_{12}:$CH_3CO_2C_2H_5$, 7:3) 675 mg (73%) of **29** was obtained as an oil; ^{1}H NMR: δ [0.98] 1.01 (d, 3H, J=6 Hz, -CH_3), 2.12–2.37 (m, 4H, -CH_2—C≡C—CH_2-), 3.30–3.37 (m, 2H, 2-CHN-), 3.38 (t, 2H, J=6.6 Hz, -CH_2OCH_2Ph), 3.98, 4.07, 4.83, 4.88 (4d, 4H, 2-NCH_2Ph), 4.45 (s, 2H, -OCH_2Ph), 5.23–5.28, 5.39–5.46 (2m, 2H, -CH=CH-), 7.22–7.34 (m, 15H, arom.); ^{13}C NMR: δ 11.91 (-CH_3), 25.72, 28.30 (-CH_2-C≡C), 45.03, 45.69 (-NCH_2Ph), 51.38, 56.13 (-CHN-), 69.37 (-CH_2O-), 72.90 (-OCH_2Ph), 126.19, 127.23 (-C≡C-), 127.58, 127.93, 127.98, 128.01, 128.33, 128.50, 128.64, 137.60, 138.30 (arom.), 160.89 (C=O); Anal. calcd for $C_{30}H_{34}N_2O_2$: C, 79.31; H, 7.48; N, 6.16. Found; C, 79.20; H, 7.57; N, 6.22.

***cis*-4-(*Z*-5-Hydroxypent-2-enyl)-5-methyl-imidazolidin-2-one (4a).** In a three-necked round-bottom flask kept at −78 °C, NH_3 (20 mL) was condensed. Absolute EtOH (3 mL) was added and the temperature raised to −30 °C. Compound **29** (175 mg, 0.38 mmol) dissolved in 3 mL THF was then added, followed by small sodium pieces (90 mg, 3.8 mat). On decolorization between two additions, NH_3 was evapd at room temperature. After addition of water, neutralization with 12 N HCl and evapn at 50 °C under reduced pressure, the residue was purified on a C18 reverse phase column (CH_3CN:H_2O, 1:9). After lyophilization, 68 mg (96%) of **4a** was obtained as a white powder; mp (dec): ^{1}H NMR (CD_3OD): δ 1.18 [1.23] (d, 3H, J=6.5 Hz, -CH_3); 2.22–2.40 (m, 4H, -CH_2—C≡C—CH_2-), 3.58 (t, 2H, J=6.7 Hz, -CH_2OH), 3.81–3.87 (m, 1H, -CHN-), 3.94–4.01 (m, 1H, J=6.4, 8 Hz, -CHN-), 5.44–5.52 (m, 1H, -CH=C-), 5.54–5.61 (m, 1H, -C=CH); ^{13}C NMR (CD_3OD): δ 15.45 (-CH_3), 28.73, 31.83 (2-CH_2-), 53.21 (-CH_2OH), 57.77, 62.33 (2-CHN-), 127.80, 130.47 (-C=C-), 165.56 (C=O). Anal calcd for $C_9H_{16}N_2O_2$: C, 58.72; H, 8.69; N, 15.21. Found: C, 58.70 H, 9.00; N, 15.00.

***cis*-4-(*Z*-5-Bromopent-2-enyl)-5-methyl-imidazolidin-2-one (30).** In a soln of **4a** (112 mg, 0.61 mmol) in anhydrous acetonitrile (1 mL) and pyridine (79 μL, 0.97 mmol) Ph_3P, Br_2 (335 mg, 0.79 mmol) was added. The mixture was stirred for 3 h at room temperature with two further additions of Ph_3P, Br_2 (335 mg) and pyridine (79 μL), after 1 and 2 h. The mixture was then filtered on silica gel and then chromatographed on a silica gel column ($CHCl_3$:CH_3OH, 9:1); 139 mg (92%) of **30** was obtained as a white solid; mp 81–83 °C; MS (EI 70 eV) ($M^{+\cdot}$): 247; ^{1}H NMR: δ 1.11 [1.17] (d, 3H, J=6.6 Hz, -CH_3), 2.08–2.14 (m, 1H, -CH—C$\underline{H}$H—C=C), 2.26–2.32 (m, 1H, -CH—CH$\underline{H}$—C=C), 2.56–2.61 (m, 2H, -C=C—CH_2-), 3.29–3.37 (m, 2H, -CH_2Br), 3.64–3.70 (m, 1H, -CHN-), 3.82–3.88 (m, 1H, -CHN-), 5.20; 5.30 (2s, 2H, 2-NH-), 5.37–5.54 (m, 2H, -CH=CH-); ^{13}C NMR: δ 15.70 (-CH_3), 28.14, 30.69, 32.26 (-CH_2-), 51.03, 55.57 (-CHN-), 127.63, 129.78 (-C=C-), 163.42 (C=O). Anal. calcd for $C_9H_{15}N_2OBr$: C, 43.72; H, 6.13; N, 11.33. Found: C, 43.89; H, 6.01; N, 11.43.

***cis*-4-(*Z*-5-Carboxypent-2-enyl)-5-methyl-imidazolidin-2-one (2a).** KCN (26 mg, 0.40 mmol) was added to **30** (50 mg, 0.20 mmol) dissolved in anhydrous DMSO (500 μL). After 3 h stirring at room temperature, DMSO was evapd under reduced pressure at 50 °C. The residue was dissolved in 1 N NaOH (3 mL) and heated at 180 °C for 3 h. The mixture was then acidified to pH 1 with 12 N HCl and purified on a C18 reverse phase column (CH_3CN:H_2O, 1:9). After lyophilization 37 mg (87%) of **2a** was obtained as a white solid recrystallized in water; mp 120–122 °C. The recrystallized product contained ~3% of the minor isomer; ^{1}H NMR (CD_3OD): δ 1.14 [1.19] (d, 3H, J=6.5 Hz, -CH_3), 2.23–2.37 (m, 6H, 3-CH_2-), 3.70–3.75 (m, 1H, J=8 Hz, -CHN-), 3.83–3.89 (dq, 1H, J=8, 6.5 Hz, -CHN-), 5.39–5.53 (m, 2H, J=10.7 Hz, -CH=CH-*cis*); ^{13}C NMR (CD_3OD): δ 15.66 (-CH_3), 24.02, 28.85, 34.72 (-CH_2-), 52.59 (-CHN-), 57.32 (-CHN-), 127.41, 131.97 (-C=C-), 166.0 (C=O), 177.02 (-COOH). Anal. calcd for $C_{10}H_{16}N_2O_3$: C, 56.58; H, 7.61; N, 13.20. Found: C, 56.60; H, 7.64; N, 13.30.

Preparation of [^{14}C]2a. To lyophilized K^{14}CN (650 μg, 10 μmol, S.A. 55.0 mCi/mmol), **30** (2.47 mg, 10 μmol) dissolved in anhydrous DMSO (100 μL) was added. The mixture was stirred for 5 h. According to TLC, the starting material was consumed. A small crystal of cold KCN was, however, added to ensure the complete consumption of **30**. After stirring for a further 2 h, the solvent was removed under vacuum at 50 °C; 1 N NaOH (3 mL) was added and the mixture refluxed for 3 h. The medium was then acidified with 12 N HCl and poured on a C18 reverse phase column (CH_3CN:H_2O, 1:9). After lyophilization, the pure product was dissolved in distilled water (10 mL) and kept at −20 °C. Titration of the obtained solution was achieved colorimetrically by using *p*-dimethylaminocinnamaldehyde.[27] [**2a**] = 690 ± 15 μM. Specific activity (SA) = 53 ± 1 mCi/mmol.

***cis*-4-(*E*-5-Hydroxypent-2-enyl)-5-methyl-imidazolidin-2-one (4b).** *n*-Butyllithium (1.6 N) in hexane (665 μL, 1.06 mmol) was added at 0 °C to a solution of the phosphonium salt[26] dried for 10 min at 80 °C under vacuum (559 mg, 1.14 mmol). The red soln was cooled at −60 °C. The aldehyde **28** (244 mg, 0.76 mmol) dissolved in THF (2 mL) was then added, followed after 25 min stirring by *n*-butyllithium (1.6 N) (523 μL, 0.84 mmol). The mixture was allowed to warm to −30 °C, treated with anhydrous $LiClO_4$ (162 mg, 1.52 mmol), kept at −30 °C for 30 min and then cooled again at −78 °C. Methanol (100 μL) was added with strong stirring and the mixture was stirred for 40 min at room temperature. After neutralization with 12 N HCl, extraction with CH_2Cl_2, the organic phase was dried and evapd and the residue was purified on silica gel (C_6H_{12}:$CH_3CO_2C_2H_5$, 7:3). A 40:60 mixture of **29** and **31** (192 mg, ~55%) was obtained as an oil. Compound **31** was identified from the ^{1}H NMR

spectrum of the mixture. ^{1}H NMR: δ 0.99 (d, 3H, J=6.4 Hz, -CH_3), 2.06–2.37 (m, 4H, -CH_2—C≡C—CH_2-), 3.22–3.48 (m, 4H, 2-CHN-, -CH_2OCH_2Ph), 3.94, 4.09, 4.72, 4.80 (4d, 4H, -NCH_2Ph), 4.39 (s, 2H, -OCH_2Ph), 5.15–5.27, 5.33–5.45 (2m, 2H, -CH=CH-), 7.15–7.32 (m, 15H, arom.).

Compounds **29** and **31** were very difficult to separate and were debenzylated as a mixture. The experimental conditions were the same as for the debenzylation of **29**. 192 mg (0.42 mmol) of the mixture was treated with 97 mg (4.2 mat) of Na. After NH_3 evapn, addition of water and neutralization with 12 N HCl, the medium was concd under vacuum at 50 °C. The resulting soln was purified by semi-prep. HPLC (C18 reverse phase column; CH_3OH:H_2O, 5:95). After lyophilization, 39 mg (28%) of **4b** was obtained as a white powder; mp (dec): ^{1}H NMR (CD_3OD): δ 1.12 [1.18] (d, 3H, J=6.5 Hz, -CH_3), 2.15–2.30 (m, 4H, -CH_2—C≡C—CH_2-), 3.59–3.63 (t, 2H, J=6.2 Hz, -CH_2OH), 3.76–3.82 (m, 1H, -CHN-), 3.90–3.97 (m, 1H, -CHN-), 5.47–5.61 (m, 2H, J=15.3 Hz, -CH=CH-); ^{13}C NMR (CD_3OD): δ 15.25 (-CH_3), 33.39, 35.77 (2-CH_2-), 52.33 (-CH_2OH), 56.55, 62.08 (2-CHN-), 129.29, 131.18 (C=C), 164.00 (C=O); HRMS: *m/z* 154 (M−CH_2O). Calcd for $C_8H_{14}N_2O$: 154.1106. Found: 154.1105.

***cis*-4-(*E*-5-Bromopent-2-enyl)-5-methyl-imidazolidin-2-one (32).** The alcohol **4b** was first transformed into the corresponding bromide **32**. The experimental conditions were the same as for the transformation of **4a** into **30**. From 20 mg of **4b**, 25 mg (92%) of the bromo derivative **32** was obtained; ^{1}H NMR: δ 1.11 (d, 3H, J=6.4 Hz, -CH_3), 2.08–2.23 (m, 2H, -CH_2-), 2.44–2.59 (m, 2H, -CH_2-), 3.30–3.39 (m, 2H, -CH_2Br), 3.62–3.68 (m, 1H, -CHN-), 3.82–3.89 (dq, 1H, J=6.6 Hz, -CHN-), 4.58, 4.66 (2s, 2H, -NH-), 5.36–5.43, 5.44–5.50 (2m, 2H, J=15.4 Hz, -CH=CH-); ^{13}C NMR: δ 15.77 (-CH_3), 32.74, 33.13 (-CH_2-), 35.63 (-CH_2Br), 50.99, 55.19 (-CHN-), 128.96, 130.94 (-CH=CH-), 162.84 (C=O); HRMS: *m/z* 99 (M−C_5H_8Br). Calcd for $C_4H_7ON_2$: 99.0558. Found: 99.0558.

***cis*-4-(*E*-5-Carboxypent-2-enyl)-5-methyl-imidazolidin-2-one (2b).** Compound **32** was treated with KCN as described for **30** and the nitrile hydrolyzed into **2b** which was isolated by C18 reverse phase chromatography with a 66% yield, as a white powder, recrystallized in water; mp 154–156 °C. The product contains ~3% of the minor isomer; ^{1}H NMR (CD_3OD): δ 1.12 [1.17] (d, 3H, J=6.5 Hz, -CH_3), 2.12–2.37 (m, 6H, 3-CH_2-), 3.67–3.73 (m, 1H, J=8 Hz, -CHN-), 3.81–3.88 (m, 1H, J=8, 6.5 Hz, -CHN-), 5.43–5.61 (m, 2H, J=15.4 Hz, -CH=CH-); ^{13}C NMR (CD_3OD): δ 15.60 (-CH_3), 29.21, 34.10, 35.13 (-CH_2-), 52.54, 57.15 (-CHN-), 128.17, 133.28 (-HC=CH-), 166.1 (C=O), 177.8 (-COOH). Anal. calcd for $C_{10}H_{16}N_2O_3$: C, 56.58; H, 7.61; N, 13.20. Found: C, 56.73; H, 7.52; N, 13.27.

Preparation of [^{14}C]2b. The experimental conditions were the same as for the preparation of **2a**. [**2b**] = 510 ± 15 μM. Specific activity (SA) = 54 ± 1.4 mCi/mmol.

Biochemical experiments

Chemicals. All chemicals were of the highest purity available and were purchased either from Sigma or Aldrich. 5-Deazaflavin was a gift of Dr E. Mulliez (Université de Grenoble, France).

Bacterial strains and plasmids. *Bacillus sphaericus* BT(250)C (*bio*R$^-$, actithiazic acid^r, 5-(2-thienyl)-valeric acid^r, 1-(2′-thenoyl)-3,3,3-trifluoroacetoner), derived from *B. sphaericus* IFO3525 (wild-type),[28] was transformed with plasmid pBHB5022[29] which bears the *B. sphaericus bio B* gene.

Protein purification. The preparation of the cell-free extract (CFE) from the transformant *B. sphaericus* BT(250)C[pBHB 5022] was performed as previously described.[7] Pure biotin synthase was obtained as previously described.[11]

Analytical methods. SDS–PAGE was performed in 12.5% gel as described by Laemmli. Proteins were stained with Coomassie Brilliant Blue. Protein concentration was measured by the method of Bradford using bovine serum albumin as a standard.

Thin-layer chromatography (TLC) was performed with Kieselgel 60F254 plates eluted with $CHCl_3$:MeOH:CH_3-COOH (90:10:5).

The amount of biotin formed in an enzymatic assay was determined on the supernatant of the incubated mixture treated with 10% aq trichloroacetic acid. A microbiological assay using *Lactobacillus plantarum* was used for quantification of biotin.[30]

For liquid scintillation counter (LSC) and linear-TLC analyzer, see above.

Visualization of the incubation products: standard assay

The reaction mixture in a final vol of 250 μL consisted of CFE (25 mg/mL), 40 μM (±) [^{14}C] substrate, 1 mM NADPH, 1 mM AdoMet, 0.5 mM cysteine, 5 mM dithiothreitol (DTT) and 40 mM tris(hydroxymethyl) aminomethane (Tris) buffer pH 8.0. The reaction mixture was incubated at 37 °C for 1 h and was stopped by heating at 100 °C for 2 min. Precipitated proteins were removed by centrifugation, washed 3 times with 200 μL of 30 mM Tris buffer pH 8.0. The total resulting supernatant was passed through a reverse phase Sep-Pak® (Waters) cartridge and eluted with 2 mL of MeOH. At this stage, 85–95% of the initial radioactivity are recovered. Aliquots of the soln obtained (each containing ~20 000 dpm radioactivity) were spotted on 20 × 20 cm TLC plates for product separation and visualization (Fig. 1).

Labelling assays

The standard reaction mixture in a final vol of 100 µL consisted of pure biotin synthase (2 mg/mL), 200 µM (±) [^{14}C] substrate and other cofactors necessary for activity in the 5-deazaflavin/irradiation system as previously described[11] with some improvements that will be published later. After incubation, the reaction mixture was passed through a PD 10 column equilibrated with 30 mM Tris buffer pH 8.0. The protein-containing fractions were concd with a Centricon 30. 100 µg of this protein was deposited in each well of an electrophoresis gel using the SDS–PAGE technique. Two gel bands comprizing 200 µg of protein were cut in the same way into 5 mm pieces, put together and eluted for 20 h in a Solulyte® (Baker) containing LSC cocktail. The radioactivity gel profiles were obtained after counting these pieces (Fig. 2).

Acknowledgements

Lactone **5** was a generous gift of the Hoffmann-La Roche Society (Basel, Nutley). This work has been supported in part by the IFSBM (Institut de Formation Supérieure Biomédicale, Villejuif, France).

References

1. Marquet, A.; Frappier, F.; Guillerm, G.; Azoulay, M.; Florentin, D.; Tabet, J. C. *J. Am. Chem. Soc.* **1993**, *115*, 2139.

2. Marti F. B. *Diss. E.T.H.* (Zürich) 1983, Nr 7236.

3. Ifuku, O.; Kishimoto, J.; Haze, S.; Yanagi, M.; Fukushima, S. *Biosci. Biotech. Biochem.* **1992**, *56*, 1780.

4. Sanyal, I.; Cohen, G.; Flint, D. H. *Biochemistry* **1994**, *33*, 3626.

5. Birch, O.M.; Fuhrmann, M.; Shaw, N.M. *J. Biol. Chem.* **1995**, *270*, 19158.

6. Ohshiro, T.; Yamamoto, M.; Izumi, Y.; Tse Sum Bui, B.; Florentin, D.; Marquet, A. *Biosci. Biotech. Biochem.* **1994**, *58*, 1738.

7. Florentin, D.; Tse Sum Bui, B.; Marquet, A.; Ohshiro, T.; Izumi, Y. *C.R. Acad. Sci. Paris* **1994**, *317*, 485.

8. Knappe, J.; Neugebauer, F. A.; Blaschkowski, H. P.; Gänzler, M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 1332.

9. Mulliez, E.; Fontecave, M.; Gaillard, J.; Reichard, P. *J. Biol. Chem.* **1993**, *268*, 2296.

10. Moss, M. L.; Frey, P. A. *J. Biol. Chem.* **1987**, *262*, 14859.

11. Mejean, A.; Tse Sum Bui, B.; Florentin, D.; Ploux, O.; Izumi, Y.; Marquet, A. *Biochem. Biophys. Res. Commun.* **1995**, *217*, 1231.

12. Conradt, H.; Hohmann-Berger, M.; Hohmann, H. P.; Blaschkowki, H. P.; Knappe, J. *Arch. Biochem. Biophys.* **1984**, *228*, 133.

13. Bianchi, V.; Eliasson, R.; Fontecave, M.; Mulliez, E.; Hoover, D. M.; Matthews, R. G.; Reichard, P. *Biochem. Biophys. Res. Commun.* **1993**, *197*, 792.

14. (a) Gerecke, M.; Zimmermann, J. P.; Aschwanden, W. *Helv. Chim. Acta* **1970**, *53*, 991; (b) Field, G. F. *J. Org. Chem.* **1978**, *43*, 1084.

15. Even, L.; Florentin, D.; Marquet, A. *Bull. Soc. Chim. Fr.* **1990**, *127*, 758.

16. Montana, M.; Nicholas, K. M.; Khan, M. A. *J. Org. Chem.* **1988**, *53*, 5193.

17. Mingotaud, A. F.; Florentin, D.; Marquet, A. *Bull. Soc. Chim. Fr.* **1992**, *129*, 457.

18. Schlosser, M.; Christmann, K. F.; Piskala, A. *Chem. Ber.* **1970**, *103*, 2814.

19. Nonhebel, D. C. *Chem. Soc. Rev.* **1993**, 347.

20. Baldwin, J. E.; Adlington, R. M.; Domayne-Hayman, B. P.; Knight, G.; Ting, H. H. *J. Chem. Soc., Chem. Commun.* **1987**, 1661.

21. Liu, K. E.; Johnson, C. C.; Newcomb, M.; Lippard, S. J. *J. Am. Chem. Soc.* **1993**, *115*, 939.

22. Baldwin, J. E.; Adlington, R. M.; Flitsch, S. L.; Ting, H. H.; Turner, N. J. *J. Chem. Soc., Chem. Commun.* **1986**, 1305.

23. Fitzpatrick, P. F.; Villafranca, J. J. *Arch. Biochem. Biophys.* **1987**, *257*, 231.

24. Baldwin, J. E.; Abraham, E. P. *Nat. Prod. Rep.* **1988**, 129.

25. De Wolf, Jr W. E.; Carr, S. A.; Varrichio, A.; Goodhart, P. J.; Mentzer, M. A.; Roberts, G. D.; Southan, C.; Dolle, R. E.; Kruse, L. I. *Biochemistry* **1988**, *27*, 9093.

26. Cubero, I. *J. Chem. Ecol.* **1991**, *17*, 1530.

27. McCormick, D. B.; Roth, J. A. *Anal. Biochem.* **1970**, *34*, 226.

28. Izumi, Y.; Kano, Y.; Inagaki, K.; Kawase, N.; Tani, Y.; Yamada, M. *Agric. Biol. Chem.* **1981**, *45*, 1983.

29. Gloeckler, R.; Ohsawa, I.; Speck, D.; Ledoux, C.; Bernard, S.; Zinsus, M.; Villeval, D.; Kisou, T.; Kamogawa, K.; Lemoine, Y. *Gene* **1990**, *87*, 63.

30.Wright, L. D.; Skeggs, H. R. *Proc. Soc. Exp. Biol. Med.* **1944**, *56*, 95.

(*Received in U.S.A.* 22 *January* 1996; *accepted* 1 *April* 1996)