Pergamon

Tetrahedron 54 (1998) 8171–8182

TETRAHEDRON

A Versatile Procedure for the Generation of Nucleoside 5′-Carboxylic Acids Using Nucleoside Oxidase

Mahmoud Mahmoudian*, Brian A.M. Rudd, Brian Cox, Chris S. Drake, Richard M. Hall, Paul Stead, Michael J. Dawson, Malcolm Chandler, David G. Livermore, Nicholas J. Turner+, Gareth Jenkins+

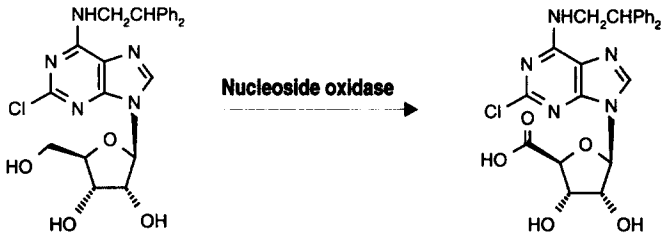
Glaxo Wellcome Research and Development, Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts, SG1 2NY, UK. +Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JJ, UK.

Received 3 March 1998; revised 7 May 1998; accepted 14 May 1998

Abstract: The nucleoside oxidase from *Stenotrophomonas maltophilia* (FERM BP-2252) has been used to generate 5′-carboxylic acid derivatives of nucleoside analogues. The enzyme, which has a surprisingly broad substrate specificity for unnatural nucleosides, has been immobilised and used at preparative scale.

Introduction

The nucleoside analogue **2** (1-[2-chloro-6-[(2,2-diphenylethyl)amino]-9*H*-purin-9-yl]-1-deoxy-β-D-ribofuranuronic acid) is a key intermediate in the synthesis of a novel group of compounds with broad anti-inflammatory properties (Figure 1). [1]



1 **2**

Figure 1

* Correspondence to: Dr Mahmoudian
✉ mm6382@glaxowellcome.co.uk

0040-4020/98/$19.00
PII: S0040-4020(98)00456-6

Although chemical oxidation of the 5′-hydroxyl of precursor **1** (2-chloro-N-(2,2-diphenylethyl)-adenosine), using transition metal oxidants (such as $KMnO_4$), has been carried out in good chemical yields, this has posed considerable problems for scale up due to the heterogeneous nature of the reaction. The lack of regioselectivity of the oxidation reaction also necessitates the use of a protection/de-protection sequence to protect the 2′/3′ hydroxyl groups, thus introducing two extra steps into the route. There are also considerable environmental and handling implications with the use of this reagent. Enzymatic oxidation of a nucleoside precursor offers potential for an improved synthesis; it is environmentally clean and obviates the need for protection of other functional groups.

We report here on an effective and practical enzymatic procedure for the preparation of **2** in high chemical yields. We have also studied the substrate specificity of nucleoside oxidase and generated a number of nucleoside 5′-carboxylates on a preparative scale.

Results and Discussion

Nucleoside oxidase is produced by *Pseudomonas* species and related Gram-negative bacteria.[2-5] Crude extracts of *Stenotrophomonas maltophilia* (formerly known as *Xanthomonas maltophilia*) exhibiting nucleoside oxidase activity have been reported to convert the 5′-hydroxyl groups of natural purine and pyrimidine nucleosides to their corresponding carboxylic acids.[2-4] The enzyme has been purified to homogeneity. It has a molecular weight of 130,000 and is composed of one each of four non-identical subunits. It has no exogenous co-factor requirements, but contains 1 mol of covalently bound FAD, 2 mol of non-haem iron, 2 mol of labile sulfides and 1 mol of haem per mol of enzyme protein, and catalyses a two step oxidation of a nucleoside *via* an aldehyde intermediate consuming one molecule of molecular oxygen.[3,4] Nucleoside oxidase has been developed for the analytical determination of nucleosides (for example in assessing food freshness),[2] but has not been reported for the preparative synthesis of nucleoside 5′-carboxylates.

We initially used crude extracts of *S. maltophilia* to oxidise selectively the 5′-hydroxyl of **1** to generate **2** on a mg scale (Figure 1). The crude extracts also cleaved the substrate slowly to release the purine base, presumably *via* a phosphorylase activity (Figure 2); this was, however, considered not to be significant when cells with a high nucleoside oxidase activity were used and crude extracts were found to be satisfactory for use in biotransformations without further purification. The enzyme was stable when stored at -20°C and retained up to 70% of its activity on storage at 4°C for 7 days, but was poorly stable at room temperature.

Crude extracts containing nucleoside oxidase were used in bioconversions with up to 20 g/L of substrate input. There was no evidence of substrate inhibition at high substrate concentrations; **1** was quickly oxidised to **2** and reactions had gone to completion within 24 hr. Oxidation of **1** was shown to go *via* a transient formation of the corresponding aldehyde intermediate to produce the carboxylic acid in high chemical yields (Figure 2).

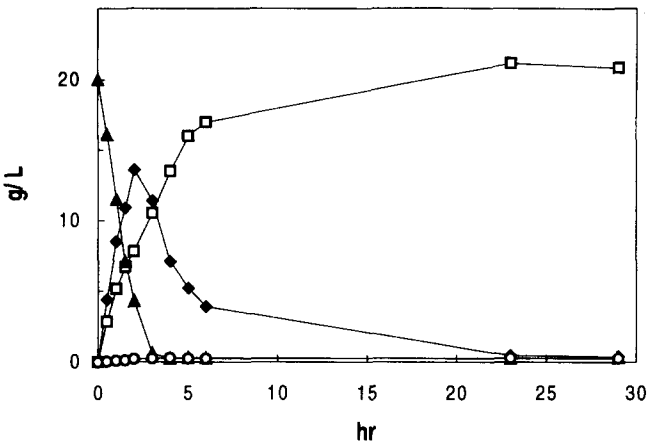


Figure 2. Reaction profile using crude extracts of *Stenotrophomonas maltophilia*. The reaction was carried out at room temperature in a magnetically-stirred flask containing 3.5 ml of a clarified crude lysate (pH 6). **1** was added as a solid (70 mg) and stirred to obtain a homogeneous suspension. Periodically, samples were removed, diluted into the mobile phase and the clarified solution assayed by hplc. **1** (▲), **2** (□), aldehyde (◆), base (○).

Due to difficulties encountered when isolating **2** from crude cell lysate we chose to investigate enzyme immobilisation to simplify downstream processing and to allow the enzyme to be re-used. Nucleoside oxidase was immobilised directly from crude homogenates of *S. maltophilia* onto Eupergit-C beads; the immobilisation efficiency was 20-40%. In free enzyme reactions, when the enzyme to substrate ratio was low, oxidation was not only slow but of limited duration and stopped before all the substrate was used. With immobilised enzyme this was also evident, even at a bead concentration of 40% (w/v) and any attempts to wash and re-use the beads were unsuccessful (data not shown).

Nucleoside oxidase is known to be able to carry out a laccase reaction which is dependent on and stoichiometric with nucleoside oxidation.[2] Working with crude extracts there was evidence that addition of a laccase substrate such as quinol increased the amount of nucleoside which could be oxidised before the reaction ceased. Therefore, we decided to investigate the effect of quinol on reactions with immobilised enzyme. Initial bioconversion rates were found to be significantly lower in the presence of 1 g/L of quinol. However, even at a substrate concentration of 20 g/L, reactions continued to completion in the presence of quinol, whereas the corresponding control reactions (without quinol) stopped at a product concentration around 15 g/L (Figure 3). This was attributed to quinol having a protective role by stabilising the enzyme during bioconversions. Consequently, the effect of quinol on enzyme re-use was investigated and it was found that the

same batch of enzyme could now be re-used for 5 cycles (data not shown). Bioconversions were scaled up to produce multi-gram quantities of **2** for further evaluation.

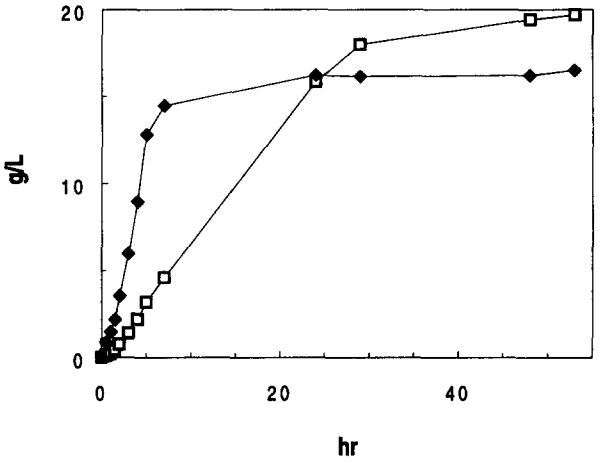


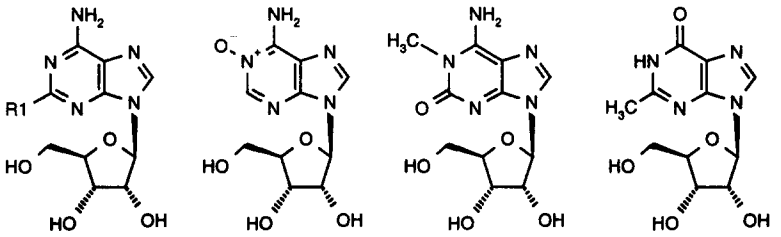
Figure 3. Effect of quinol on bioconversions using the immobilised enzyme. The reaction (5 ml) was carried out in 50 mM potassium phosphate buffer (pH 6). **1** (100 mg) was added as a solid and stirred at room temperature to obtain a homogeneous suspension. The reaction was started by the addition of washed immobilised beads at 40% (w/v). At intervals, samples were removed, cleared of enzyme beads and analysed by hplc. Reactions were carried out either in the presence (□) or absence (◆) of quinol at 1 g/L.

The substrate specificity of nucleoside oxidase towards natural nucleosides has been described previously.[2,5] The enzyme accepts purine and pyrimidine nucleosides having ribose, deoxyribose or arabinose as a sugar moiety but does not oxidise the sugar in the absence of a base.[2,5] We have extended these studies to investigate the effect of the enzyme on ribosides of unnatural purine bases and on carbocyclic nucleosides.

As the natural purine nucleosides (adenosine, guanosine, inosine, xanthosine) are all good substrates it appears that the enzyme is quite tolerant of changes in the purine base, accepting either amino or carbonyl functionality at both positions 2 and 6. It was, however, surprising to find that **1**, with the bulky diphenylethylamino group at the 6-position, was also a good substrate. We have shown further that the enzyme is tolerant of different functionality at the 2-position including chloro (**3**) or phenylethyl amino (**4**), Figure 4. Furthermore, the nitrogen at the 1-position can be modified to the N-oxide (**5**) or methylated in the case of methyl isoguanosine (**6**). Interestingly, in

the inosine series, with 2-methylinosine (**7**) as a substrate, the reaction tended to stop at the 5′-aldehyde intermediate and was not further oxidised.

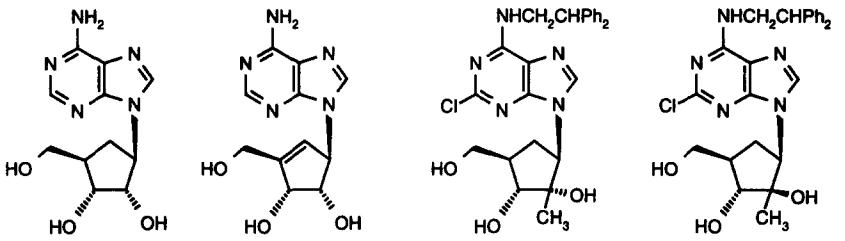
Carbocyclic nucleosides such as aristeromycin (**8**) and neplanocin A (**9**) were also selectively oxidised to give the 5′-carboxylates (Figure 5), whereas oxidation of the carbocyclic analogue of **1** (**10**) resulted in a mixture of the 5′-carboxylate **11** and the conjugated aldehyde **12** (Figure 6). The enzyme did not, however, accept a methyl group at the 2′-position of the carbocyclic moiety (**13**, **14**, Figure 5)[6] or the 2′,3′ acetonides of any of the natural nucleosides.



3 R1= Cl
4 R1=$NHCH_2CH_2(C_6H_5)$

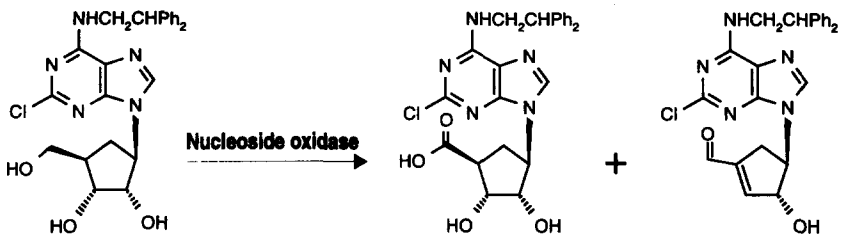
5 **6** **7**

Figure 4



8 **9** **13** **14**

Figure 5



10 11 12

Figure 6

Conclusions

The synthetic utility of the nucleoside oxidase from *Stenotrophomonas maltophilia* was exploited to produce the 5′-carboxylates of several purine nucleoside analogues, including the carbocyclic nucleosides aristeromycin and neplanocin A on a preparative scale. This has formed the basis of a scaleable process for the generation of nucleoside 5′-carboxylic acid derivatives. The enzyme has a surprisingly wide substrate specificity toward unnatural nucleosides especially in the base moiety.

Experimental

General procedures: The substrates for the enzymatic oxidation reactions were obtained from commercial sources (as stated) or prepared within the Medicinal Chemistry and Development Chemistry Departments at Glaxo Wellcome. Eupergit-C was obtained from Rohm Pharma, Darmstadt, Germany. All other chemicals were of analytical grade (BDH or Fisons). Analytical Reagent or hplc grade solvents were used throughout (Rathburn Chemicals Ltd).

^{1}H-NMR spectra were recorded on either a Bruker AMX500 (500 MHz), a Varian VXR400 (400 MHz), a Bruker AC 250 (250 MHz) or a Bruker AC 300 (300 MHz) spectrometer. ^{13}C-NMR spectra were recorded on either a Bruker AC 250 (62.9 MHz), a Bruker AC 300 (75.5 MHz) or a Bruker AMX500 (100.62 MHz) spectrometer. ESI-MS spectra, in positive mode (ESI +ve), were obtained on a Finnigan Mat TSQ-700 mass spectrometer fitted with its own source. Mass measurements (electrospray) were obtained on a VG Autospec machine.

Analytical methods: The extent of bioconversions was determined using reverse phase hplc (Hewlett Packard). This system was also used to monitor product isolation. Where necessary, prior to analysis, proteins and debris were removed from samples by centrifugation (10,000*g* for 10 min) or by filtration (Millipore, 0.45 μm sterile filters). The filtrate was diluted 10-fold with the mobile phase and 50 μl was injected onto the column. The hplc system used a Hypersil BDS-C_{18}

column (15 x 0.46 cm) and mobile phase of 10 mM ammonium acetate containing 40% (v/v) acetonitrile with a flow rate of 1 ml/ min. Detection wavelength was 280 nm.

Growth, immobilisation and bioconversion procedures: Small scale cultures of *S. maltophilia* (FERM BP-2252) were grown routinely at 25°C in 50 ml volumes of medium (Yeast extract, 25 g/L; glucose, 30 g/L; K_2HPO_4, 1 g/L; KCl, 1 g/L; $MgSO_4$, 0.5 g/L) in shake flasks (250 rev/min, 5 cm throw). Fermenter cultures (50 L) were inoculated using five Florence flasks each containing 200 ml of an 8 hr old nutrient broth (2% v/v). For larger scale cultivations, 450-500 L fermentations were inoculated from two 8 hr old 5 L seed fermenters grown as above (2% v/v). The fermenter (Biolafitte, 780 L nominal capacity), which was fitted with three Rushton impellers, was agitated at 250-500 rev/min and aerated at 250 L/min. Fermentation was allowed to proceed for 15-48 hr before the cells were harvested using a Sharples AS16-PY continuous centrifuge and stored at -20°C until required.

Typically, up to 2 kg (wet weight) of *S. maltophilia* cell paste was suspended to give a 20% (w/v) suspension in lysis buffer (either potassium phosphate, 50 mM; EDTA, 1 mM; DTT, 1 mM; PMSF, 1 mM; pH, 6.0 or potassium phosphate, 50 mM; pH, 6.0 or sodium acetate, 50 mM; pH, 5.6) and disrupted by passing the suspension through a Manton-Gaulin homogeniser operating at 8,000 psi, or through a Dynomill (flow rate, 20 L/hr; speed, 3200 rev/min; bead size, 0.25-0.5 μm). Homogenisation was carried out three times, with cooling of the suspension to 5°C after each pass through the Manton-Gaulin or cooling to -20°C through the Dynomill, to obtain the crude extract. Small scale cell disruption was carried out using a MSE Soniprep 150 ultrasonicator fitted with a 3mm exponential microprobe. The sonication was carried out in 5 x 45 seconds bursts with cooling intervals. The enzyme in the crude extract was immobilised directly, without centrifugation, onto Eupergit-C (10 g dry beads/ g protein) and the mixture was left to stand at room temperature with occasional mixing to ensure adequate infiltration. After approximately 7 days, the beads were washed with cold buffer (Tris-HCl, 100 mM; EDTA, 1 mM; DTT, 1 mM; NaCl, 0.5 M; p-hydroxybenzoic acid ethyl ester, 500 ppm; pH 7.5) then stored in the same buffer minus NaCl at 4°C.

Bioconversions using either free or immobilised nucleoside oxidase were carried out in magnetically-stirred glass vials (5 ml working volume) or in shake flasks (500 ml working volume). The reaction mixtures contained 0.5-20 g/L of nucleoside substrate, either added as a solid or in methanol [10% (v/v) final concentration], in 50 mM potassium phosphate (pH 6.0), or in 50 mM sodium acetate (pH 5.6) buffer and were incubated at either ambient temperature or at 25°C. Quinol was added at 1 g/L where appropriate. Bioconversions with the immobilised enzyme were started by the addition of washed beads to 40% (w/v). Periodically, 0.5 ml samples were removed and diluted appropriately into the mobile phase, the beads were filtered and the filtrate assayed by hplc.

Enzymic preparation of the oxidation product of 1: To 5L of a clarified crude lysate of *S. maltophilia* (pH 6) was added 10 g of **1**[7] and the reaction mixture was incubated overnight at 25°C.

Upon completion of reaction, acetonitrile (1 vol) was added, stirred, and left to stand at ambient temperature for 2 hr. Precipitated solids were pelleted by centrifugation (2500 rpm x 30 min) and the supernatant was taken and evaporated to remove organic solvent. The solution was pumped through a bed of Amberchrom CG161 resin (500 ml bed) and the resin was washed with 20% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). The product was eluted with 80% (v/v) acetonitrile. The product-containing eluate was evaporated to 700 ml, the pH was adjusted to 2.0 (H_3PO_4) and the solution was stored at 4°C for 72 hr. The precipitated solid was harvested by centrifugation (2500 rpm x 30 min), washed with water and dried *in vacuo* over P_2O_5 to give 10.5 g of crystalline **2**. ESI (+ve) *m/z* 496, 498 $(M+H)^+$ in a ratio of 3:1, (Found: MH^+, 496.139049. $C_{24}H_{23}ClN_5O_5$ requires MH^+, 496.138772); ^{1}H-NMR (DMSO-d_6) mixture of rotamers (≈ 3:1), data for major rotamer, δ 13.11 (1H, bs, 5′-OH), 8.43 (1H, t, J = 5.5 Hz, 6-NH), 8.42 (1H, s, H-8), 7.34 (4H, d, J = 7.5 Hz, o-Ph) 7.31 (4H, t, J = 7.5 Hz, m-Ph), 7.20 (2H, t, J = 7.5 Hz, p-Ph), 5.96 (1H, d, J = 6.5 Hz, 1′-H), 5.83 (1H, bs, 3′-OH), 5.78 (1H, d, J = 5.5 Hz, 2′-OH), 4.58 (1H, t, J = 8.0 Hz, CHPh), 4.47 (1H, m, 2′-H), 4.41 (1H, d, J = 2.5 Hz, 4′-H), 4.30 (1H, m, 3′-H), 4.09 (2H, m, CH_2). ^{13}C-NMR (100.62 MHz, DMSO-d_6) δ172.3 (C), 155.2 (C), 153.5 (C), 150,0 (C), 142.5 (C), 128.8 (CH), 128.4 (CH), 126.8 (CH), 118.8 (C), 86.8 (CH), 83.0 (CH), 74.5 (CH), 73.6 (CH), 49.8 (CH), 44.8 (CH_2).

Enzymic preparation of the oxidation product of inosine: To 30 ml of the clarified crude lysate was added 50 mg of inosine (Sigma) and the mixture was incubated at 25°C for 20 hr. When reaction was completed, the clarified mixture was pumped through a column containing Macro-Prep High Q resin (acetate form; 40 ml bed). The resin was washed with water then eluted sequentially with increasing concentrations of acetic acid and formic acid in water. The product was contained in the 2M formic acid eluate. This fraction was freeze dried to yield the 5′-carboxylic acid product as a white powder (39 mg). ESI (+ve) *m/z* 283 $(M+H)^+$, (Found: MH^+, 283.067731. $C_{10}H_{11}N_4O_6$ requires MH^+, 283.067859); ^{1}H-NMR (DMSO-d_6) δ 13.18 (1H, bs, 5′-OH), 12.45 (1H, bd, J = 3.5, NH), 8.42 (1H, s, H-8), 8.10 (1H, d, J = 3.5 Hz, H-2), 6.03 (1H, d, J = 6.5 Hz, 1′-H), 5.84 (1H, bs, OH), 5.70 (1H, bs, OH), 4.49 (1-H, dd, J = 6.5, 4.5 Hz, 2′-H), 4.43 (1H, d, J = 2.5 Hz, 4′-H), 4.31 (1H, dd, J = 4.5, 2.5 Hz, 3′-H). ^{13}C-NMR (100.62 MHz, DMSO-d_6) δ172.3 (C), 156.8 (C), 149.4 (C), 146.5 (CH), 138.7 (CH), 124.2 (C), 87.3 (CH), 83.0 (CH), 74.7 (CH), 73.6 (CH).

Enzymic preparation of the oxidation product of xanthosine: To 850 ml of the clarified crude lysate was added 10 g of xanthosine (Sigma) and the mixture was incubated at 25°C overnight. Upon completion of reaction, methanol (1 vol) was added and left to stand at 4°C overnight after which the precipitated solids were pelleted by centrifugation (12,000 rev/min x 30 min). The supernatant was taken and evaporated to a volume of 90 ml. The pH was adjusted to 3.0 (conc. HCl) and propan-2-ol (2 vol) was added. After standing at 4°C for 72 hr, solid was filtered off (Whatman No 1), washed with propan-2-ol and dried *in vacuo* over P_2O_5 to obtain 6.6 g of the 5′-carboxylic acid product. ESI (+ve) *m/z* 299 $(M+H)^+$; ^{1}H-NMR (DMSO-d_6) δ 16.08 (1H, bs,

5′-OH), 10.61 (1H, s, NH), 7.71 (1H, s, 8-H), 5.74 (1H, d, J = 8.5 Hz, 1′-H), 5.58 (1H, bs, 3′-OH), 5.47 (1H, bs, 2′-OH), 4.17 (1H, s, 4′-H), 4.10 (1H, m, 2′-H), 4.03 (1H, d, J = 4.0 Hz, 3′-H).

Enzymic preparation of the oxidation product of 3: To 600 ml of the clarified crude lysate was added 2 g of 2-chloroadenosine **3** (Sigma) and the mixture was incubated at 25°C for 5 hr. Upon completion of reaction, the mixture was stirred with methanol (1 vol), stood at ambient temperature for 2 hr, then filtered (Whatman No. 1) to remove precipitated solids. The filtrate was evaporated to remove organic solvent, the pH was adjusted to 2.0 (conc. HCl) and the solution was stored at 4°C for 72 hr. The product was filtered (Whatman No. 1), washed with acidified water and the crystalline solid was dried *in vacuo* over P_2O_5 to obtain 2.05 g of the 5′-carboxylic acid product. ESI (+ve) *m/z* 316, 318 $(M+H)^+$ in a ratio of 3:1, (Found: MH^+, 316.044592. $C_{10}H_{11}ClN_5O_5$ requires MH^+, 316.044871); ^{1}H-NMR (DMSO-d_6) δ 13.22 (1H, bs, 5′-OH), 8.47 (1H, s, 8-H), 7.88 (2H, bs, 6-NH_2), 5.98 (1H, d, J = 6.5 Hz, 1′-H), 5.85 (1H, d, J = 5.0 Hz, 3′-OH), 5.70 (1H, d, J = 6.0 Hz, 2′-OH), 4.50 (1H, m, 2′-H), 4.43 (1H, d, J = 2.5, 4′-H), 4.31 (1H, m, 3′-H). ^{13}C-NMR (100.62 MHz, DMSO-d_6) δ172.3 (C), 157.1 (C), 153.6 (C), 151.2 (C), 139.8 (CH), 118.1 (C), 87.1 (CH), 82.9 (CH), 74.4 (CH), 73.6 (CH).

Enzymic preparation of the oxidation products of 4[8], adenosine N[1]-oxide (5)[9] and 7[10]: These reactions were carried out at analytical scale in magnetically-stirred glass vials (5 ml working volume) containing 1 mg/ml of each of the substrates. Bioconversions were started at 25°C by the addition of clarified crude lysate (up to 5 ml at pH 6) and monitored by hplc and mass spectrometry for several days. The identity of products was evidenced by LC-MS-MS; i.e. an increase of +14 mass units shown by fragmentation to occur in the sugar portion.

Enzymic preparation of the oxidation product of 6: To 150 ml of the clarified crude lysate was added 100 mg methyl isoguanosine **6**[11] and the mixture was incubated at 25°C for 20 hr. Upon completion of the reaction, the mixture was stirred with methanol (1 vol) for 1 hr then centrifuged at 2500 rev/min for 90 min. The supernatant was taken and evaporated to a volume of 100 ml then passed through a column containing Amberlite XAD-16 resin (50 ml bed). The resin was washed with water and adsorbed components were eluted with 50% (v/v) acetonitrile in H_2O (2 bed vol). The cloudy eluate was filtered through a bed of Dikalite and the filtrate was taken and evaporated to *ca* 6 ml. The pH was adjusted to 2 (H_3PO_4) and the solution left to crystallise at 4°C for 48 hr. Crystalline product (28 mg) was filtered off (Whatman No. 1) and dried *in vacuo*. ESI (+ve) *m/z* 312 $(M+H)^+$, (Found: MH^+, 312.094316. $C_{11}H_{14}N_5O_6$ requires MH^+, 312.094408); ^{1}H-NMR (DMSO-d_6) δ 15.56 (1H, bs, 5′-OH), 8.52 (2H, bs, 6-NH_2), 8.01 (1H, s, 8-H), 5.84 (1H, bs, 3′-OH), 5.82 (1H, d, J = 7.0 Hz, 1′-H), 5.62 (1H, d, J = 5.5 Hz, 2′-OH), 4.37 (1H, d, J = 1.5 Hz, 4′-H), 4.31 (1H, m, 2′-H), 4.20 (1H, m, 3′-H) 3.78 (3H, s, 1-CH_3).

Enzymic preparation of the oxidation product of aristeromycin (8) [12]: To 200 ml of the clarified crude lysate was added 1 g of aristeromycin and the mixture was incubated at 25°C overnight. Upon completion of reaction, the mixture was stirred with methanol (1 vol) for 1 hr then centrifuged at 2500 rev/min for 90 min. The supernatant was taken and evaporated to dryness and freeze-dried overnight. The crude material was purified initially by anion exchange chromatography [Dowex-1X8 resin, elution with 5% (v/v) methanol] followed by preparative HPLC [Spherisorb ODS1 10μm; 20 x 250 mm; eluant 1% (v/v) CH_3CN in 50 mM ammonium formate pH 3.0 at 20 ml/min]. Lyophilisation gave aristeromycin 5'-carboxylic acid as a white solid (800 mg, 76%). ESI (+ve FAB) *m/z* 280 $(M+H)^+$ [Found: MH+, 280.10324. $C_{11}H_{14}N_5O_4$ requires MH, 280.10458). ^{1}H-NMR (DMSO-d_6) δ 8.26 (1H, s, adenine-H), 8.11 (1H, s, adenine-H), 7.19 (2H, s, NH_2), 4.65-4.76 (1H, m, 1'-H), 4.31 (1H, dd, J = 5.0, 8.5Hz, 3'-H), 4.14-4.17 (1H, m, 2'-H), 2.64-2.70 (1H, m, 4'-H), 2.32-2.44 (1H, m, 6'-H), 2.11-2.23 (1H, m, 6'-H). ^{13}C-NMR (62.9 MHz, DMSO-d_6) δ 176.2 (C), 156.1 (C), 152.3 (CH), 150.0 (C), 140.0 (CH), 119.3 (C), 75.5 (CH), 72.2 (CH), 59.0 (CH), 49.4 (CH), 30.2 (CH_2).

Enzymic preparation of the oxidation product of neplanocin A (9) [13]: To 200 ml of the clarified crude lysate (pH 5.6) was added 200 mg of neplanocin A and the mixture was incubated at 25°C. After 2 days, the reaction had only progressed to 25% conversion, a further 200 ml of the clarified crude lysate was added and the mixture was incubated for a further 2 days. At this stage reaction was 50% complete and the mixture was stirred with methanol (1 vol) for 1 hr then centrifuged at 2500 rev/min for 90 min. The supernatant was taken and evaporated to dryness and freeze-dried overnight. Purification of the crude material as described above for aristeromycin 5'-carboxylic acid gave, after lyophilisation, neplanocin A 5'-carboxylic acid as a white solid (120 mg, 57%). ESI (+ve) *m/z* (EI) 277 (Found: M^+, 277.08095. $C_{11}H_{11}N_5O_4$ requires M, 277.08110). ^{1}H-NMR (DMSO-d_6) δ 8.15 (1H, s, adenine-H), 8.12 (1H, s, adenine-H), 7.20 (2H, s, NH_2), 6.81 (1H, d, J = 1.5 Hz, 6'-H), 5.54 (1H, d, J = 6.5 Hz, 1'-H), 4.60 (1H, d, J = 5.5 Hz, 3'-H), 4.44 (1H, dd, J = 5.5, 6.5 Hz, 2'-H). ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ165.0 (C), 156.0 (C), 152.3 (CH), 149.7 (C), 142.2 (CH), 140.3 (CH), 139.3 (C), 119.2 (C), 76.8 (CH), 71.0 (CH), 64.2 (CH).

Enzymic preparation of the oxidation product of 10: To 120 ml of the clarified crude lysate was added 100 mg of **10**[14] and the mixture was incubated at 25°C for 20 hr. Upon completion of reaction, the mixture was pumped through a column containing reverse phase silica (Bond-Elut 1g; Spherisorb 5 μm C_{18}). The resin was washed with water and adsorbed components were eluted with 80% (v/v) acetonitrile in H_2O. Concentrated eluate was subjected to hplc on 5 μm Kromasil C_8 (25 x 2.5 cm) as follows: Solvent A: 35% (v/v) acetonitrile, 50 mM $NH_4H_2PO_4$ with 1 ml/L H_3PO_4 added. Solvent B: 70% (v/v) acetonitrile in 50 mM $NH_4H_2PO_4$ with 1 ml/L H_3PO_4 added. Gradient profile: 100% A to 100% B in 20 min at 25 ml/min. Relevant fractions were evaporated to remove organic solvent, adsorbed onto Spherisorb C_{18} (Bond-Elut 1 g), washed with water and eluted with acetonitrile, then freeze-dried to yield the 5'-carboxylate **11** (7.5 mg) and aldehyde **12** (2.5 mg) as white powders. ESI (+ve) *m/z* of **11**, 494, 496 $(M+H)^+$ in a ratio of 3:1, (Found: MH^+,

494.158855. $C_{25}H_{25}ClN_5O_4$ requires MH^+, 494.159507); ESI (+ve) *m/z* of **12**, 460, 462 $(M+H)^+$ in a ratio of 3:1; ^{1}H-NMR of **11**: (DMSO-d_6) mixture of rotamers (≈ 4:1), data for major rotamer, δ 12.28 (1H, s, 5′-OH), 8.29 (1H, t, J = 5.5 Hz, NH), 8.22 (1H, s, 8-H), 7.35 (4H, d, J = 7.5 Hz, o-Ph) 7.31 (4H, t, J = 7.5 Hz, m-Ph), 7.20 (2H, t, J = 7.5 Hz, p-Ph), 5.15 (1H, d, J = 5.0 Hz, 3′-OH), 5.13 (1H, d, J = 6.0 Hz, 2′-OH), 4.67(1H, m, 1′-H), 4.58 (1H, t, J = 8.0 Hz, CHPh), 4.25 (1H, m, 2′-H), 4.17 (1H, m, 3′-H), 4.08 (2H, dd, J = 8.0, 5.5 Hz, NCH_2), 2.77 (1H, td, J = 8.5, 2.5 Hz, 4′-H), 2.41 (1H, dt, J = 13.0, 8.5 Hz, 6′-H), 2.11 (1H, ddd, J = 13.0, 10.0, 8.5 Hz, 6′-H). ^{13}C-NMR (100.62 MHz, DMSO-d_6) δ175.0 (C), 152.3 (C), 150.5 (C), 149.5 (C), 140.0 (C), 128.5 (CH), 128.1 (CH), 126.5 (CH), 118.0 (C), 74.8 (CH), 71.2 (CH), 58.0 (CH), 44.2 (CH_2), 29.3 (CH_2). ^{1}H-NMR of **12**: (DMSO-d_6) mixture of rotamers (≈ 4:1), data for major rotamer, δ 9.81 (1H, s, 5′-H), 8.35 (1H, t, J = 5.5 Hz, NH), 8.26 (1H, s, 8-H), 7.35 (4H, d, J = 7.5 Hz, o-Ph) 7.31 (4H, t, J = 7.5 Hz, m-Ph), 7.21 (2H, t, J = 7.5 Hz, p-Ph), 7.03 (1H, s, 3′-H), 5.92 (1H, d, J = 6.5 Hz, 2′-OH), 5.35 (1H, t, J = 6.5 Hz, 2′-H), 4.81 (1H, m, 1′-H), 4.58 (1H, t, J = 8.0 Hz, CHPh), 4.08 (2H, dd, J = 8.0, 5.5 Hz, NCH_2), 3.00 (1H, dd, J = 16.0, 9.5 Hz, 6′-H), 2.83 (1H, dd, J = 16.0, 8.5 Hz, 6′-H).

Acknowledgements. The authors thank Dr Phil Sidebottom and Dr Steve Freeman for NMR analyses and Mr Nick Taylor and Mr Ian Davidson for Mass spec. analyses. Contributions from, Dr Paul Ravenscroft and his team are also gratefully appreciated.

References and notes

1. Gregson, M.; Ayres, B.E.; Ewan, G.B.; Ellis, F.; Knight, J. *PCT Int. Appl.*, *WO* 9417090, **1994**.
2. Isono, Y.; Hoshino, M. *US Pat. Appl.* 5,156,955, **1992**.
3. Isono, Y.; Sudo, T.; Hoshino, M. *Agric. Biol. Chem.* **1989**a, *53*, 1663-69.
4. Isono, Y.; Sudo, T.; Hoshino, M. *Agric. Biol. Chem.* **1989**b, *53*, 1671-77.
5. Misaki, H.; Ikuta, S.; Matsunia, K. *US Pat. Appl.* 4,385,112, **1983**.
6. Compounds **13** & **14** were synthesised from **8** in 4 steps: the 3′-OH/5′-OH TIPS protection of **8** using dichlorotetraisopropylsiloxane in pyridine, followed by oxidation of the 2′ hydroxyl group to the ketone using Moffatt conditions (DCC/orthophosphoric acid in DMSO), reaction with methylmagnesium iodide in diethyl ether and separation of the diastereomeric mixture of tertiary alcohols and finally deprotection using potassium fluoride dihydrate/18-crown-6 in THF.
7. For the synthesis of **1** see; Bristol, J.A.; Trivedi, B.; Moos, W.H. *Eur. Pat. Appl.*, *EP* 139358, **1985** & Ayres, B.E.; Gregson, M.; Ewan, G.B.; Keeling, S.E.; Bell, R. *PCT Int. Appl.*, *WO* 9602553, **1996**.

8. For the synthesis of **4** see; Marumoto, R.; Yoshioka, Y.; Miyashita, O.; Shima, S.; Imai, K.; Kawazoe, K.; Honjo, M. Chem. Pharm. Bull. **1975**, *23*, 759-74 & Hutchison, A.J.; Francis, J.E. *Eur. Pat. Appl., EP* 323807, **1989**.
9. Adenosine N-oxide (**5**), was obtained from Sigma.
10. 2-Methylinosine (**7**) was synthesised in one step from 5-amino-1-β-D-ribofuranosyl-4-imidazolecarboxamide using the method of Ajinomoto Co., Inc. *Neth. Appl., NL* 6600317, **1966**.
11. Methyl isoguanosine (**6**) was synthesised in one step from 5-amino-1-β-D-ribofuranosyl-4-imidazolecarboxamide using the method of Chern, J. W.; Lin, G. S.; Chen, C. S.; Townsend, L. B. *J. Org. Chem.* **1991**, *56*, 4213-18.
12. Aristeromycin was produced by fermentation of *Streptomyces citricolor*, see Kusaka, T.; Yamamoto, H.; Shibata, M.; Muroi, M.; Kishi, T.; Mizuno, K. *J. Antibiotics* **1967**, *21*, 255-63.
13. Neplanocin A was produced by fermentation of a mutant strain of *Streptomyces citricolor*, see; Hill, J.M.; Jenkins, G.N.; Rush, C.P.; Turner, N.J.; Willets, A.J.; Buss, A.D.; Dawson, M.J.; Rudd, B.A.M. *J. Am. Chem. Soc.* **1995**, *117*, 5391-92.
14. For the synthesis of **10** see; Ayres, B.E.; Gregson, M.; Ewan, G.B.; Chandler, M. *PCT Int. Appl., WO* 9602543, **1996**.