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Studies on the Exchange of Valine-Oxygen During The Biosynthesis of δ-(*L*-α-Aminoadipoyl)-*L*-cysteinyl-*D*-valine.

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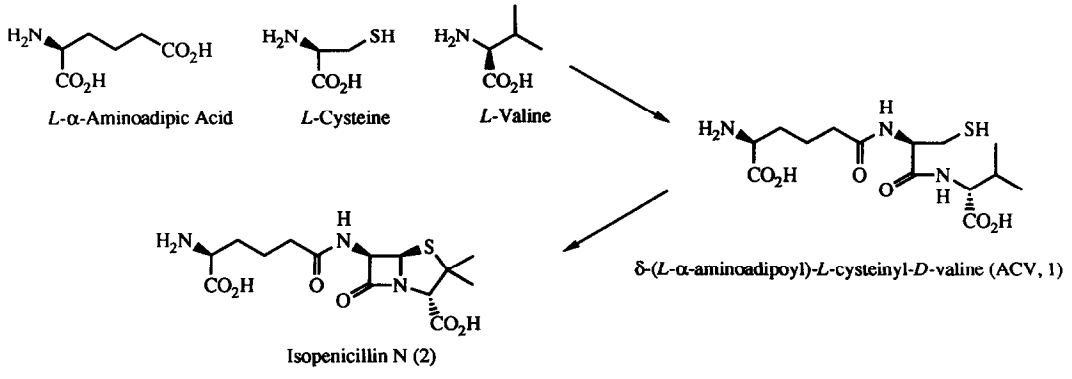
Key Words : *Cephalosporium acremonium*; biosynthesis; δ-(*L*-α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (ACV); [$^{18}O_2$]-valine incorporation; penicillin.

Abstract : Incorporation of [4-$^{2}H_6$,$^{18}O_2$]-valine into δ-(*L*-α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (ACV), by intact cells of *Cephalosporium acremonium*, demonstrated the intracellular exchange of one and both valine oxygen atoms. Incubation of [$^{18}O_2$]-valine with the purified ACV synthetase from *C. acremonium* gave exclusive incorporation of a single ^{18}O label into ACV, consistent with the effectively non-reversible formation of a covalent valinoyl-ACV synthetase intermediate under *in vitro* conditions.

The first step in the biosynthesis of penicillins and cephalosporins involves the enzymatic condensation of *L*-α-aminoadipic acid, *L*-cysteine, and *L*-valine with concomitant stereochemical inversion of the valine α-centre, generating the tripeptide δ-(*L*-α-aminoadipoyl)-*L*-cysteinyl-*D*-valine (ACV, 1)[1]. The tripeptide so formed is cyclized by an oxidative desaturation process to give isopenicillin N (2), the first formed penicillin (Scheme 1)[1].

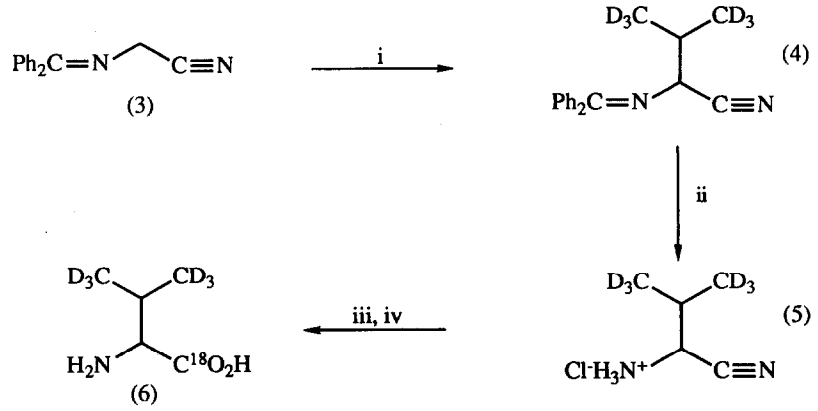


Scheme 1

Two studies relating to the fate of valine carboxyl oxygen atoms during penicillin biosynthesis have been reported in the literature[2,3]. *In vivo* studies conducted by Delderfield *et al* with *Penicillium chrysogenum* have shown the exchange of one or both valine oxygen atoms from [$^{18}O_2$]-valine during the formation of penicillin V[2]. However, problems with the interpretation of such data arise due to the possibility of valine carboxyl-oxygen exchange processes unrelated to the β-lactam biosynthetic pathway. Additionally, it is not possible to distinguish on the basis of these experiments whether oxygen exchange takes place prior to or during tripeptide formation, or during or following penicillin formation. No conclusions can be drawn from analysis of the [$^{16}O_2$]-penicillin formed in this type of experiment due to the potential interference by the endogenous biosynthesis of ACV (1) from unlabelled precursors. Subsequent studies[3] employing incubations of unlabelled ACV tripeptide (2) with cell free extracts of *Cephalosporium acremonium* in $^{17}O/^{18}O$ enriched water showed no incorporation of $^{17}O/^{18}O$ into the penicillin product, suggesting that valine-oxygen exchange takes place either during or prior to ACV tripeptide (1) formation. We now report *in vivo* and *in vitro* studies pertaining to the fate of valine-oxygen during ACV (1) biosynthesis[4,5].

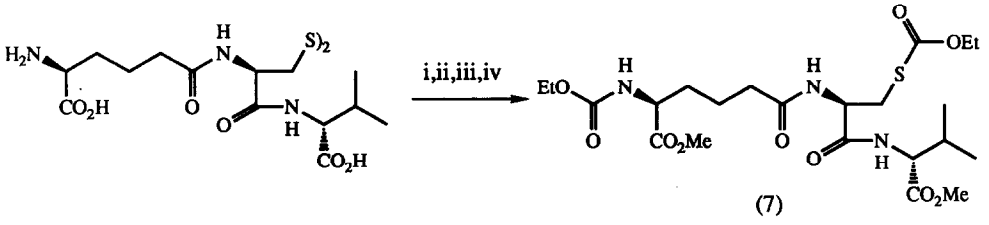
Initially *in vivo* studies were conducted. Two experimental features were introduced in order to assist in determining the extent of exchange of valine oxygen during ACV (1) biosynthesis. Firstly, a β-lactam negative strain of *C. acremonium*, the Takeda N-2 mutant[6], which has no active penicillin-forming enzyme, was selected for these studies to enable direct examination of the incorporation of valine into ACV (1), rather than into the downstream penicillin. Secondly, to overcome the complications of endogenous ACV (1) biosynthesis in whole cell studies, [4-$^2H_6$, $^{18}O_2$]-valine was synthesised, so that the fate of the exogenous precursor could be followed more discretely.

[4-$^2H_6$, $^{18}O_2$]-Valine was synthesised according to Scheme 2 in an overall yield of 70% from labelled 2-bromopropane using literature methods, and modifications thereof. Thus, alkylation of nitrile (3) with 1,3-[$^2H_3$]-2-bromopropane using the catalytic phase transfer method of O'Donnell and Eckrich[7] gave substituted nitrile (4). Selective imine hydrolysis with 1M hydrochloric acid at room temperature yielded 2-amino-3-[$^2H_3$]-methyl-4-[$^2H_3$]-butyronitrile hydrochloride salt (5), which on refluxing with $H_2{}^{18}O$ saturated with dry hydrogen chloride gas followed by ion-exchange chromatography gave *D,L*-[4-$^2H_6$, $^{18}O_2$]-valine (6).



Scheme 2. Reagents: i, $(CD_3)_2CHBr$, $PhCH_2N^+Et_3Cl^-$, 50% aq. NaOH, 0°C to room temp. over 3h, then room temp., 12h; ii, 1M HCl, 25°C, 12h; iii, $H_2{}^{18}O$, HCl (g) (sat.d), reflux, 12h; iv, ion-exchange

The racemic[8] material (6) was fed at a level of 12.5mg/ml to a culture of the Takeda N-2 mutant of *C. acremonium* as soon as the stationary phase of growth was established. Fermentation was continued for 3 days at which point the culture was sonicated to liberate intracellular ACV (1) and subjected to centrifugation and filtration to provide a clear lysate from which ACV (1) disulphide and valine were recovered by reverse phase h.p.l.c.. ACV (1) disulphide obtained in this manner was reduced with dithiothreitol (DTT) and chemically derivatized to give its *N,S*-diethoxycarbonyldimethyl ester derivative (7)[3] (Scheme 3). The recovered valine and ACV derivative (7) were then examined by chemical ionization mass spectrometry (Table 1).



Scheme 3. Reagents: i, DTT, H_2O, pH 8.0; ii, $(EtO)_2CO$; iii, H_3O^+, then AcOEt extraction; iv, CH_2N_2,

Table 1 : NH_3 Desorption chemical ionization mass spectrometry results for ACV derivatives and recovered valine from feeding [4-$^2H_6$, 1-$^{18}O_2$]-valine (6) to whole cells of *Cephalosporium acremonium*, N-2 mutant.

Compound		$^{16}O_2$		$^{16}O^{18}O$			$^{18}O_2$	
1.Synthetic	*m/z*	123	124	125	126	127	128	129
[4-$^2H_6$, 1-$^{18}O_2$]-Valine (6)	% obs.	5	9	7	53	13	100	10
	m/z	541	542	543	544	545	546	547
2.ACV derivative (7)	% obs.	5	77	32	100	35	19	5
	m/z	123	124	125	126	127	128	129
3.Recovered Valine (6)	% obs.	1	14	5	61	11	100	6

The data obtained indicates substantial loss of both one and two oxygen atoms during the conversion of the labelled valine (6) to ACV (1). The possibility of $^{16}O_2$ material arising from endogenous ACV biosynthesis can be precluded in these experiments, but one is still left facing the possibility that this material arises through exchange processes unrelated to ACV biosynthesis. Control experiments (Table 2) where synthetic δ-(*L*-α-aminoadipoyl)-*L*-cysteinyl-*D*-[$^{18}O_2$]-valine (8) disulphide was substituted for [4-$^2H_6$, $^{18}O_2$]-valine (6) demonstrated the lack of extensive extracellular exchange of valinyl oxygens from the tripeptide, and a culture fluid only experiment (which had been separated from mycelia grown to the stationary phase) with [$^{18}O_2$]-valine (9) gave no labelled ACV (1), demonstrating that conversion of valine to ACV is an intracellular process.

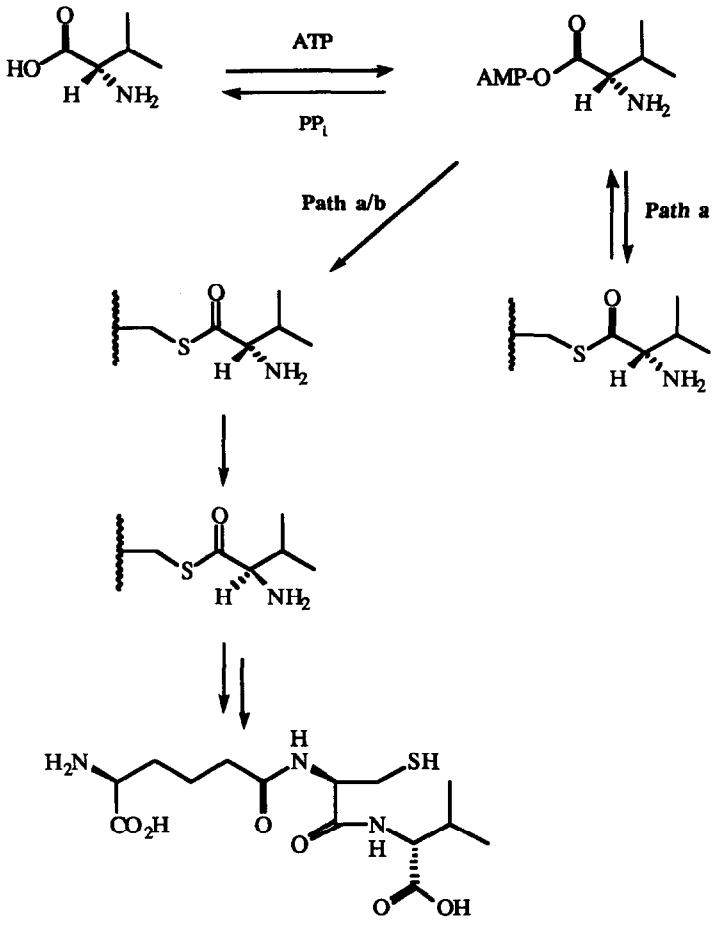
Table 2 : NH_3 Desorption chemical ionization mass spectrometry results: extracellular exchange control experiment using [$^{18}O_2$]-ACV (8)

Compound		$^{16}O_2$		$^{16}O^{18}O$		$^{18}O_2$		
1. [$^{18}O_2$]-ACV derivative (7)	*m/z*	536	537	538	539	540	541	542
(extracellular control experiment)	% obs.	1	1	29	12	100	30	4
2. Recovered	*m/z*	536	537	538	539	540	541	542
ACV derivative (7)	% obs.	45[a]	14[a]	38[a]	13	100	29	12
(culture fluid only experiment)								

[a] These values are affected by endogenous ACV production

Taken as a whole these results probably indicate that an intracellular exchange of one and both valinyl oxygen atoms occurs prior to ACV excretion from intact cells of the Takeda N-2 mutant of *C. acremonium*.

In vitro studies on the biosynthesis of ACV have proved troublesome for a number of years due to the lability of the enzymatic system involved. Banko *et al* [9] have shown that ACV formation is catalyzed by a single enzyme, unlike the structurally related tripeptide glutathione (*L*-γ-glutamyl-*L*-cysteinyl-glycine), which is formed by two separate enzymes[11]. The ACV forming enzyme, ACV synthetase (ACVS), has now been isolated from a number of sources, following the initial report by van Liempt *et al* [12] of the purification to apparent homogeneity of ACVS from *Aspergillus nidulans*. The genes encoding ACV synthetase from several organisms have been cloned[13] and give rise to predicted molecular weights for the ACVSs in excess of 400KDa. Current indications suggest that ACV synthetase belongs to the ATP-dependent non-ribosomal peptide synthetase class of enzymes[14]; sequence homologies have been reported between ACVS and other peptide synthetases[13] and ACVS has been shown to contain a pantotheinyl group[17]. Such enzymes have been proposed to operate via a thiol-template mechanism with a pantotheine 'swinging arm' transferring activated amino acids to sites on the enzyme capable of catalysing peptide bond formation[15]. Amino acid activation is proposed to involve a two-step process with initial amino-acyl adenylate formation followed by transfer of the amino-acyl moiety onto the enzyme in the form of a thioester link to a cysteine residue[12,15] (no such cysteine residues have been positively identified as yet). Whilst no bond is ultimately formed to the valine carboxyl group of ACV, there is evidence for activation of this amino acid as an amino-acyl adenylate, and subsequent valinoylation of ACV synthetase[12]. It is possible that such activation is required to facilitate epimerization of the valine α-centre, probably at the putative thioester stage (Scheme 4). It has been shown that aminoacyl-adenylate formation is a reversible process through incorporation of radiolabel from ^{32}P pyrophosphate into ATP, but the reversibility of subsequent thioester formation has not been investigated. One would expect that for reversible thioester formation (Scheme 4, path a), incubation of [$^{18}O_2$]-valine with ACVS would result in formation of ACV with loss of one or both ^{18}O labels. In contrast, irreversible thioester formation would result in the ACV bearing a single ^{18}O label (Scheme 4, path b). Clearly *in vivo* studies cannot address this point due to the possible interference of valine carboxyl oxygen exchange processes unrelated to ACV formation.



ACV, 1

Scheme 4. The possible fates of valine carboxyl oxygen atoms during ACV biosynthesis. Path a : exchange of >1 ^{18}O label; Path b : exchange of a single ^{18}O label

Purification of sufficient ACVS activity to allow mechanistic studies has not been possible until recently[5]. In our hands, high levels of ACVS activity have now been obtained from *C. acremonium* C0728 which has enabled the semi-synthetic scale (*i.e.* 1mg range) *in vitro* production of ACV (1) from its precursor amino acids[5]. We have been able to confirm by ^1H n.m.r. (500MHz), for the first time, the identity of the tripeptide formed from *in vitro* incubations with aminoadipate, cysteine, and valine. In addition, the advent of electrospray mass spectrometry has allowed the isotopic analysis of underivatized ACV (1) on a small scale (<10μg). Previously chemical derivatization of ACV (1), which is low yielding on a small scale, has been required to obtain chemical ionization mass spectra[3]. As a result of such developments we are now able to report[5] in full *in vitro* studies on the exchange of valine carboxyl oxygen during ACV (1) formation.

ACVS was partially purified form *Cephalosporium acremonium* C0728 using a modification[16] of the literature procedure[17]. Following cell lysis and DNA precipitation, the resulting protein solution was subjected to

ammonium sulphate fractionation and gel filtration chromatography. At this level of purity the ACVS obtained routinely had a specific activity[18] in excess of 700 pkat/g. Labelled valine (9), together with other required co-factors and co-substrates was incubated with ACVS at 27°C for 4 hours and the reaction worked up as described in the experimental section. ACV (1) formed and recovered valine (9) were purified from the reaction mixture by reverse phase h.p.l.c. and subjected to isotopic analysis by electrospray mass spectrometry (Table 3).

Table 3 : Electrospray mass spectrometry results for ACV and recovered valine from the incubation of *D,L*-[$^{18}O_2$]-valine with ACV synthetase from *Cephalosporium acremonium*.

Compound			$^{16}O_2$		$^{16}O^{18}O$		$^{18}O_2$	
	m/z	117	118	119	120	121	122	123
1.Synthetic *D,L*-[$^{18}O_2$]-Valine (9)	% obs.	-	5	-	10	-	100	7
	m/z	363	364	365	366	367	368	369
2.ACV formed[a]	% obs.	15	7	10	100	22	8	4
	m/z	117	118	119	120	121	122	123
3.Recovered Valine	% obs.	1	1	2	3	-	100	6
	m/z	117	118	119	120	121	122	123
4.Recovered Valine[b]	% obs.	1	-	-	7	-	100	6
	m/z	117	118	119	120	121	122	123
5.Recovered Valine[c]	% obs.	-	2	-	8	2	100	6

[a] ACV (1) was analyzed as its free thiol form following DTT reduction

[b] Incubation with *L*-α-aminoadipic acid and cysteine omitted

[c] Incubation with AMP and pyrophosphate (3mol/mol valine) but with *L*-α-aminoadipic acid and cysteine omitted

Within experimental error, the data obtained indicates exclusive loss of a single ^{18}O label during ACV (1) formation and very little or no exchange of label in the recovered valine. In addition, incubation of [$^{18}O_2$]-valine (9) with ACVS in the absence of aminoadipic acid and cysteine, but otherwise under the same conditions also resulted in little or no loss of ^{18}O label (Table 3, entry 4). Repeating this latter experiment with added AMP and pyrophosphate (Table 3, entry 5) resulted in little or no loss of ^{18}O label in the recovered valine suggesting that formation of a putative ACVS-valine thioester is not reversed by the presence of AMP and pyrophosphate.

The *in vitro* exchange of ^{18}O label from [$^{18}O_2$]-valine observed in this study is consistent with the effectively non-reversible formation of a reactive covalent intermediate, possibly a thioester, between the valine carboxyl group and ACV synthetase during ACV formation. In contrast, Vater *et al*[19] have reported that for the peptide-forming Gramicidin synthetase, formation of amino-acylenzyme thioesters is a freely reversible process. They have shown that it is possible to release radioactive thioester bound amino acids from the enzyme by treatment with adenosine-5'-monophosphate (AMP) and pyrophosphate, the by-products of amino-acyl adenylate formation .

This report details the first *in vitro* mechanistic studies on ACV synthetase using isotopically labelled amino acids. Further studies to investigate the structure of the putative covalent intermediate and the mechanism of the complex enzymatic process catalyzed by this enzyme are in progress.

EXPERIMENTAL

Where possible, all reagents used were analytical grade. Petrol refers to the petroleum ether fraction boiling between 30-40°C, and was distilled prior to use. Trifluoroacetic acid and anisole were freshly distilled prior to use. ^{1}H n.m.r. spectra were recorded either at 200MHz on a Varian Gemini 200 spectrometer or at 500MHz on a Bruker AM 500 spectrometer; data for samples in D_2O was acquired with HOD suppression. Chemical shifts are quoted with respect to residual protonated solvent as an internal reference. Multiplicities are recorded as (br) broad, (s) singlet, (d) doublet, or (m) multiplet. Infrared spectra were recorded on a Perkin-Elmer 1750 IR FT spectrometer, only selected resonances are reported, and are recorded as (s) strong, (m) medium, (w) weak. Mass spectra were recorded on a VG Micromass 30F spectrometer using ammonia desorption chemical ionization or on a VG BIO Q triple quadropole mass spectrometer equipped with an electrospray interface using the electrospray technique. Flash column chromatography was carried out using Sorbsil C60 40/60 flash silica gel. Thin layer chromatography was performed on aluminium-backed plates coated with Merck silica gel $60F_{254}$. Compounds were visualized with a 5% (w/v) solution of dodecamolybdophosphoric acid in ethanol. H.p.l.c. was performed using either a Waters or Gilson h.p.l.c. system with A_{214} detection. Aqueous solvents were prepared from Milli-Q water (Millipore) and h.p.l.c. grade methanol (Rathburn) was used throughout. All h.p.l.c. solvents were filtered through a 0.22μM filter and degassed prior to use.

Synthesis of Labelled Compounds

D,L-[4-$^2H_6$, $^{18}O_2$]-valine (6)

2-Amino-3-[$^2H_3$]-methyl-4-[$^2H_3$]-butyronitrile (2)[20] (74mg, 750μmol) in [^{18}O]-water (1ml of 98.4 atom%) was stirred under argon and dry hydrogen chloride gas was passed into the reaction vessel for 5min. The mixture was refluxed under argon overnight, cooled, and passed through an ion-exchange resin (Dowex 80-400, acetate form) with water as eluant. Ninhydrin positive fractions were pooled and evaporated to dryness to give, as a white solid, *D,L*-[4-$^2H_6$, $^{18}O_2$]-valine (**6**) (85mg, 93%); ν_{max} (KBr disc) 3160-2900 (w), 2565 (w), 2210 (w), 2115 (w), 1580 (s), 1495 (s), 1410 (s), 1360 (m), 1310 (s) cm^{-1}; δ_H (500MHz; D_2O) 2.07 {1H, br s, CHC<u>H</u>$(CD_3)_2$}, 3.43 {1H, d, J=5Hz, C<u>H</u>CH$(CD_3)_2$}; m/z (DCI, NH_3) 128 (MH^+, 10%), 78 (M-C[$^{18}O_2$]H, 100%).

Synthesis of δ-(*L*-α-Aminoadipoyl)-*L*-cysteinyl-*D*-[$^{18}O_2$]-valine (8)

D,L-[$^{18}O_2$]-valine (**9**) was prepared as described for compound (**6**) except that 2-amino-3-methyl-butyronitrile[20] was used in place of nitrile (**4**). *D,L*-[$^{18}O_2$]-valine (**9**) (69mg, 80%); ν_{max} (KBr disc) 3160-2950 (w), 1570 (s), 1500 (s), 1410 (m), 1360 (m), 1305 (s) cm^{-1}; δ_H (500MHz; D_2O) 0.82 (3H, d, J=6.5Hz, CHC<u>H</u>$_3$), 0.88 (3H, d, J=6.5Hz, CHC<u>H</u>$_3$), 2.07-2.15 (1H, m, C<u>H</u>Me_2), 3.44 (1H, d, J=5Hz, C<u>H</u>CHMe_2); m/z (DCI, NH_3) 122 (MH^+, 100%), 72 (M-C[$^{18}O_2$]H, 64%).

D,L-[$^{18}O_2$]-Valine (**9**) (60mg, 496μmol) was dissolved in water with stirring and *p*-toluenesulphonic acid (98mg, 515μmol) added. Stirring was continued for 5min and the mixture was freeze-dried. Acetonitrile (10ml) was added to the resulting suspension, followed dropwise by diphenyldiazomethane (*ca.*100mg, 520μmol) in ether (*ca.*1ml) until a pink colouration persisted. The solution was stirred for 10min and the reaction quenched by addition of a few drops of acetic acid (10% v/v) in ether. Solvent was removed *in vacuo* and the resulting material partitioned between ether and dilute aqueous hydrochloric acid. The organic layer was separated and the aqueous layer washed with ether. The pH of the aqueous phase was adjusted to 8 by addition of sodium

bicarbonate solution (saturated) and the organic material was extracted into ether. The combined organic extracts were dried over sodium sulphate and concentrated to dryness *in vacuo* to give the crude benzhydryl ester (**10**) (94mg, 65%); δ_H (200MHz, $CDCl_3$) 0.84 (3H, d, J=6.5Hz, $CHC\underline{H}_3$), 0.98 (3H, d, J=6.5Hz, $C\underline{H}CH_3$), 1.60 (2H, br s, NH_2), 2.04-2.25 (1H, m, $C\underline{H}Me_2$), 3.44 (1H, d, J=5.0Hz, $C\underline{H}CHMe_2$), 6.95 (1H, s, $C\underline{H}Ph_2$), 7.23-7.42 (10H, m, aromatic). The crude benzhydryl ester was used without further purification.

D,L-[$^{18}O_2$]-valine benzhydryl ester (**10**) (94mg, 45μmol) and [(5*S*)-5-*N*-*p*-methoxybenzyloxycarbonylamino-5-*p*-methoxybenzylcarbonylpentanamido]-*S*-*p*-methoxybenzyl-*L*-cysteine (**11**)[20] (208mg, 32μmol) were dissolved in dichloromethane (12ml). 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (77mg, 32μmol) was added and stirring under argon continued overnight. The resulting solution was poured into sodium bicarbonate solution (saturated) and organic material extracted with ethyl acetate. The combined organic extracts were washed with dilute hydrochloric acid and brine, dried over sodium sulphate, and concentrated to dryness *in vacuo*. The resulting diastereomeric mixture of protected tripeptides was separated by flash chromatography (silica, petrol/ethyl acetate 1:1), the required *L,L,D*-isomer being detected by t.l.c. comparison with an authenic unlabelled sample (silica, petrol/ethyl acetate 3:1, R_f 0.65). Fractions containing the desired stereoisomer were combined and concentrated to dryness to give the fully protected tripeptide (**12**) (98mg, 36%); δ_H ($CDCl_3$) 0.78 (3H, d, J=7.0Hz, $CHC\underline{H}_3$), 0.88 (3H, d, J=7.0Hz, $CHC\underline{H}_3$), 1.53-2.38 (7H, br m, $(C\underline{H}_2)_3$ and $SC\underline{H}_2Ar$), 4.28-4.80 (3H, m, 3 x $C\underline{H}NH$), 5.02 (2H, s, $ArC\underline{H}_2O$), 5.55 (1H, br d, J=7.0Hz, N$\underline{H}$), 6.35 (1H, br d, J=7.0Hz, N$\underline{H}$), 6.80-6.98 (7H, m, $C\underline{H}Ph_2$ and aromatic), 7.20-7.41 (16H, m, aromatic); m/z (field desorption) 937 (M^+).

The fully protected tripeptide (**12**) (100mg, 110μmol) was dissolved in trifluoroacetic acid (1ml) containing anisole (100μl) and was heated at reflux overnight under nitrogen. The flask was cooled and trifluoroacetic acid removed by azeotropic distillation with toluene. The resulting material was partitioned between ethyl acetate and water. The aqueous phase was separated and freeze-dried to give the title compound (**8**) quantitiatively (40mg); δ_H (D_2O) 0.73-0.80 {6H, m, $CH(C\underline{H}_3)_2$}, 1.46-1.81 (4H, 2 x m, $C\underline{H}_2C\underline{H}_2C\underline{H}_2CO$), 1.98-2.06 (1H, m, $C\underline{H}Me_2$), 2.20-2.26 (2H, m, $C\underline{H}_2CO$), 2.67-2.79 (2H, m, AB part of ABX, $C\underline{H}_2S$), 3.66-3.73 (1H, m, $C\underline{H}NH_2$), 4.09 (1H, d, J=6.5Hz, $C\underline{H}CHMe_2$), 4.38 (1H, X of ABX, $CHCH_2S$); m/z (FAB) 368 (MH^+, 100), 369 (25), 370 (12). Mass spectrum of *N,S*-diethoxycarbonyl, dimethylester derivative of (**8**)[3]: m/z (DCI, NH_3) 540 (MH+, 66%), 541 (20%), 542 (9%), 543 (3%).

IN VIVO FEEDING EXPERIMENTS

Culture conditions

Sterilisation of fungal growth media was carried out at 121°C for 20 minutes at 15 p.s.i.. *Cephalosporium acremonium*, Takeda N-2 mutant, was stored in 30% glycerol (2ml total volume) at -70°C. Samples of this culture (2ml/seed flask) were used to inoculate seed medium (30ml/250ml flask) consisting of sucrose (30g), beef extract (Oxoid L29) (15g), corn steep liquor (5g), and calcium carbonate (1.5g) per litre, pH 6.8. The seed culture was incubated at 27°C for 72h and used to inoculate a main growth culture. The main growth medium was prepared from two components, the sugar component being prepared and sterilized separately, and added to the other component prior to inoculation.

Component A: *D,L*-methionine (4g), $(NH_4)_2SO_4$ (10g), salt solution 1 (10ml), and salt solution 2 (180ml), per litre, pH 7.3 [salt solution 1: $FeNH_4(SO_4)_2.6H_2O$ (1g) in 50ml water. Salt solution 2: $MnSO_4.4H_2O$ (0.2g),

$CuSO_4.5H_2O$ (0.05g), $ZnSO_4.7H_2O$ (0.2g), $MgSO_4.7H_2O$ (2.4g), $Na_2SO_4.10H_2O$ (11.5g), K_2HPO4 (104g), KH_2PO4 (102g), $CaCl_2.2H_2O$ (0.5g), made up to 900ml with H_2O].

Sugar solution: sucrose (36g) and glucose (27g) in 250ml water.

Mycelial growths were routinely conducted in 500ml conical flasks containing component A (60ml) and sugar solution (20ml). Seed culture (72h old) was used to inoculate (5ml/flask) the main growth flasks and incubations were carried out on a gyratory shaker (200r.p.m., eccentric throw 5cm) until the stationary phase of growth was reached (approx 72h). Mycelial growth was monitored from A_{550} measurements on 1 in 20 dilutions of the culture.

Incubation with labelled valine

A sample of 72h old main growth culture was transferred to a sterile universal bottle containing labelled *D,L*-valine (25mg) and the culture incubated for a further 72h at 27°C. The culture was transferred to a pyrex vessel and sonicated on ice to effect mycelial lysis. Particulate material was removed by centrifugation (20,000 x g, 20min) and protein precipitated by addition of methanol to a final concentration of 66% (v/v). The precipitated protein was removed by centrifugation (20,000 x g, 20 min) and the supernatant lyophilized. The resulting solid was redissolved in Milli-Q water and ACV (1) disulphide and valine isolated by h.p.l.c.

Recovery of ACV and valine

ACV disulphide and valine were isolated by standard reverse phase h.p.l.c. on an octadecylsilane column employing isocratic elution (4.6 x 250mm column, eluted at 1ml/min). The column was eluted with NH_4HCO_3 (25mM) and methanol (4:1) for ACV disulphide recovery, which typically gave a retention time of 6.0min. Valine was recovered by employing isocratic elution with aqueous NH_4HCO_3 (25mM), giving a retention time of 3.6min.

Derivatization of ACV for mass spectrometry

The derivatization of ACV as its *N,S*-diethoxycarbonyl, dimethyl ester (7) was conducted as described in the literature[3].

IN VITRO EXPERIMENTS

ACV synthetase was partially purified from *C.acremonium* CO728 using a modification of the literature procedure[17]. Full details will be described elsewhere. Following gel filtration chromatography, the enzyme preparations used in this study typically showed a specific activity[18] in excess of 700pkat/g.

Incubations with ACV Synthetase

Incubation of [$^{18}O_2$]-valine with ACV synthetase was typically carried out in a total volume of 3ml containing: $MgCl_2$ (32mM), *L*-α-aminoadipic acid (2.5mM), *L*-cysteine (2.5mM), racemic [$^{18}O_2$]-valine (5mM), DTT (3mM), ATP (20mM), and ACV synthetase (approximately 1.0pkat total activity) in TRIS buffer (50mM), pH 7.5 . Following incubation at 27°C for 4h, the reaction was quenched by precipitation of protein by the addition of an equal volume of acetone. Solid was removed by centrifugation (20,000 x g, 20min) and solvent removed *in vacuo*. The resulting glycerol-based solution was diluted with water and lyophilized (glycerol is carried through from the enzyme preparation and could not be conveniently removed without significant loss of enzyme activity).

Sample preparation in this manner resulted in almost complete conversion of ACV to its disulphide form. ACV disulphide and valine were then recovered by h.p.l.c. as described earlier.

Preparation of samples for electrospray mass spectrometry

ACV samples were analysed as the reduced, thiol form of the tripeptide following reduction of neutral samples of ACV with DTT. Valine samples were analysed directly without further treatment.

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References and Notes:

1. Baldwin, J.E.; Abraham, E.P. *Nat. Prod. Rep.* **1988**, *5*, 129-145.
2. Delderfield, J.S.; Mtetwa, E.; Thomas, R.; Tyobeka, T.E. *J. Chem. Soc., Chem. Commun.* **1981**, 650-652.
3. Adlington, R.M.; Aplin, R.T.; Baldwin, J.E.; Chakravati, B.; Field, L.D.; John, E-M.M.; Abraham, E.P.; White, R.L. *Tetrahedron* **1983**, *39*, 1061-1068 and references cited therein.
4. Baldwin, J.E.; Adlington, R.M.; Bird, J.W.; Schofield, C.J. *J. Chem. Soc., Chem. Commun.* **1989**, 1615-1616.
5. Baldwin, J.E.; Field, R.A.; Schofield,C.J. *J. Chem. Soc., Chem. Commun.* **1991**, 1531-1532.
6. Baldwin, J.E.; Wan, T.S. *Tetrahedron* **1981**, *37*, 1589-1595 and references cited therein.
7. O'Donnell, M.J.; Eckrich, T.M. *Tetrahedron Lett.* **1978**, *47*, 4625-4628.
8. Only *L*-valine is thought to be converted to ACV in broken cell experiments with *C. acremonium*[9] but it is known that both *L*- and *D*-forms contribute to the intracellular pool of *L*-valine when added to intact cells of *P. chrysogenum*[10], although incorporation of *D*-valine into the mycelium is much slower.
9. Banko, G.; Demain, A.L.; Wolfe, S. *J. Am. Chem. Soc.* **1987**, *109*, 2858-2860.
10. Stevens, C.M.; De Long, C.W. *J. Biol. Chem.* **1958**, *230*, 991-999.
11. Meister, A. *Science* **1973**, *180*, 33-39.
12. van Liempt, H.; von Döhren, H.; Kleinkauf, H. *J. Biol. Chem.* **1989**, *264*, 3680-3684.
13. a. Smith, D.J.; Burnham, M.K.R.; Bull, J.H.; Hodgson, J.E.; Ward, J.M.; Browne, P.; Brown, J.; Barton, B.; Earl, A.J.; Turner, G. *EMBO J.* **1990**, *9*, 741-747, for instance.
b. Smith, D.J.; Earl, A.J.; Turner, G. *EMBO J.* **1990**, *9*, 2743-2750.
14. Kleinkauf, H.; von Döhren,H. in *Biochemistry of Peptide Antibiotics*; Kleinkauf, H; von Döhren, H. Eds.; de Gruyter, Berlin, 1990.
15. Lipmann, F. *Science* **1971**, *173*, 875-884.
16. Full experimental details will be reported elsewhere.
17. Baldwin, J.E.; Bird, J.W.; Field, R.A.; O'Callaghan, N.M.; Schofield, C.J.; Willis, A.C. *J. Antibiot.* **1991**, *44*, 241-248.
18. Specific activities are reported as pmol ACV formed / s / gram protein and were determined according to the literature method[17].
19. Vater, J.; Mallow, N.; Gerhardt, S.; Gadow, A.; Kleinkauf, H. *Biochemistry* **1985**, *24*, 2022-2027.
20. Baldwin, J.E.; Adlington, R.M.; Crouch, N.P.; Schofield, C.J.; (in part) Aplin, R.T. *Tetrahedron*, submitted for publication.