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Enzymes of Secondary Metabolism in Microorganisms

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1 Introduction

The production of microbial secondary metabolites has been a traditional focus of interest in the pharmaceutical and agrochemical industries for many years, particularly in the manufacture of antibiotics and agrochemicals by fermentation methods. Prominent amongst the microorganisms used in this field are the actinomycetes (*e.g.* tetracyclines, macrolides, aminoglycosides, and β-lactams, from *Streptomyces*), the β-lactam synthesizing fungi (*Penicillium* and *Cephalosporium*), as well as the peptide forming *Bacilli*. The Streptomycetes have a remarkable capacity for the production of secondary metabolites of diverse structural types, and are by far the richest source of natural products with antibiotic or other biological activities.

There has recently been a resurgence of interest in microbial chemistry and biochemistry, due in part to the increased appreciation of the potential of biological catalysts, both for the degradation of unwanted man-made compounds in the environment and for achieving useful transformations in organic synthesis. The antibiotic-producing microorganisms clearly have a well oiled enzymic machinery in place for the synthesis of complex molecules under mild conditions. The gamut of reactions catalysed by these enzymes of secondary metabolism (*i.e.* enzymes that are not required for growth in pure culture) is remarkable, and their substrate specificities often appear to be much less strict than the enzymes of primary metabolism. However, until recently, few had been purified to homogeneity for detailed mechanistic and structural studies. The reasons for this are easily recognized, and include the frequent instability, or lack of free intermediates at key steps in secondary metabolic pathways, the very low concentration in wild type organisms of many of the relevant enzymes, and the interrelated problem of devising sensitive and specific assays. It is an exciting prospect, therefore, that molecular genetics now offers a way round some of these difficulties, by providing access to the primary structures of these proteins *via* the DNA sequences of their cloned genes, as well as to the overproduction of the enzymes by genetic engineering methods.

From chemical and enzymological viewpoints, an important objective for the future will be to relate the synthetic operations needed to assemble a particular class of chemical structures to the mechanisms of action, specificity, structure, and organization of the biosynthetic enzymes, and to understand how this information is encoded and interpreted by the cellular machinery. Good progress

has been made already, both in the isolation of biosynthetic structural genes and in the understanding of their regulatory mechanisms. In the near future rDNA methods will permit comparative studies of structural genes (*e.g.* β-lactam-forming enzymes from *Streptomyces* and *Cephalosporium*, or the various macrolide polyketide synthases from Streptomycetes) and provide routes for their manipulation. A further prospect arises, that of combining within a single cell enzymes of different pathways in order to generate novel hybrid metabolites, a feat achieved for the first time recently by the transfer of genetic information between strains producing different polyketide antibiotics. The rational manipulation of secondary metabolism, however, will require a far more extensive knowledge of these processes, and remains some way off.

This review highlights recent research which focuses on these enzymic aspects of secondary metabolite formation in microorganisms, and also introduces the important contributions now being made by the molecular genetic approach. Already it is clear that the area holds great promise for the future development of new biological catalysts, as well as for the discovery of novel and interesting chemistry.

2 β-Lactam Antibiotics

A. IPNS Synthase.—The isopenicillin-N synthase (IPNS) from *Cephalosporium acremonium* is an enzyme of molecular weight 38 416, which catalyses the oxidative conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (LLD-ACV) into isopenicillin-N, this being a central reaction in the pathway to the clinically important penicillin and cephalosporin antibiotics[1] (Scheme 1). The enzyme has been purified not only from *C. acremonium*,[2,3] but also from *Penicillium chrysogenum*[4] and *Streptomyces clavuligerus*.[5] In the presence of O_2 and Fe^{2+}, the enzyme removes four hydrogen atoms from its substrate in a stereospecific manner,[1,6,7] and forms the azetidinone and thiazolidine rings of isopenicillin-N (Scheme 2). There is good evidence available from competitive mixed-label kinetic isotope effect measurements,[1b,8] as well as from the inhibitory action of the substrate analogue[9] (1), that the formation of an enzyme-bound monocyclic

[1] For recent reviews see: (*a*), J. E. Baldwin, in 'Proceedings of the 3rd International Symposium on Recent Advances in the Chemistry of β-Lactam Antibiotics, 1984,' ed. A. G. Brown and S. M. Roberts, Royal Society of Chemistry, London, 1985; (*b*) J. A. Robinson and D. Gani, *Nat. Prod. Rep.*, 1985, **2**, 293.

[2] C. P. Pang, B. Chakravarti, R. M. Adlington, H. H. Ting, R. L. White, G. S. Jayatilake, J. E. Baldwin, and E. P. Abraham, *Biochem. J.*, 1984, **222**, 789.

[3] I. J. Hollander, Y.-Q. Shen, J. Heim, A. L. Demain, and S. Wolfe, *Science*, 1984, **224**, 610.

[4] F. R. Ramos, M. J. Lopez-Nieto, and J. F. Martin, *Antimicrob. Agents Chemother.*, 1985, **27**, 380.

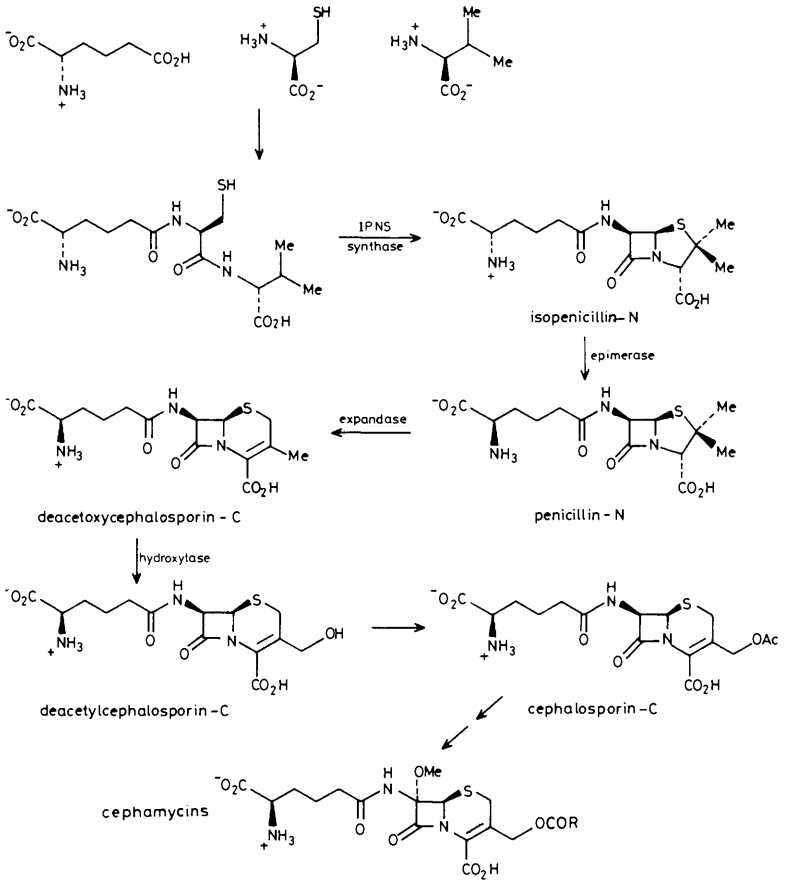
[5] S. E. Jensen, B. K. Leskiw, L. C. Vining, Y. Aharonowitz, D. W. S. Westlake, and S. Wolfe, *Can. J. Microbiol.*, 1986, **32**, 953.

[6] J. E. Baldwin, R. M. Adlington, N. G. Robinson, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1986, 409.

[7] J. E. Baldwin, R. M. Adlington, B. P. Domayne-Hayman, H. H. Ting, and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1986, 110.

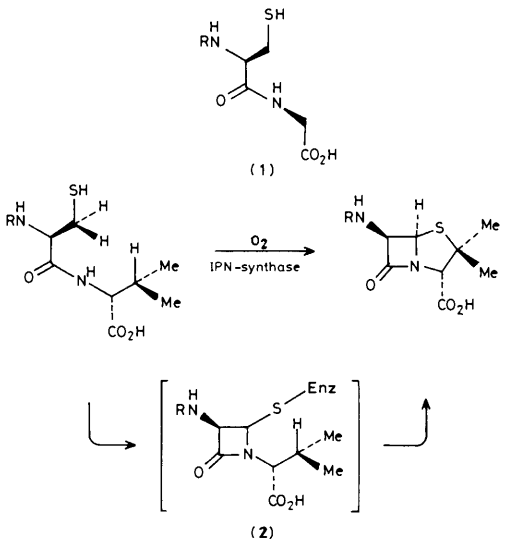
[8] J. E. Baldwin, R. M. Adlington, S. E. Moroney, L. D. Field, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1984, 984.

[9] J. E. Baldwin, E. P. Abraham, C. G. Lovel, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1984, 902.

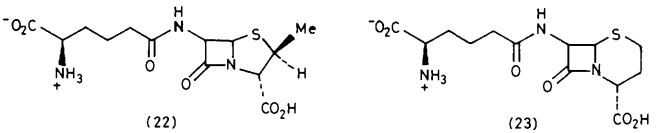


Scheme 1

β-lactam such as (2) represents the first irreversible step in the transformation. The reaction occurs with complete retention of the cysteinyl 3-*pro-R* hydrogen and complete loss of the 3-*pro-S* hydrogen,[6] and the stereospecificity remains unperturbed even when the energetically more demanding cleavage of a carbon–deuterium bond, rather than a carbon–protium bond, is presented to the enzyme in a stereospecifically deuterated substrate. The mechanism of the β-lactam ring formation is not yet clear, but it must be linked with the *formal* reduction of dioxygen to H_2O_2 at the active site of the protein. It is, however, in the second stage of the transformation, with the closure of the thiazolidine ring, that the substantially more exergonic reduction of an activated oxygen to water takes place. It is here also that remarkable new insights into the chemistry surrounding closure of the five-membered ring have come, from experiments in which the



Scheme 2



enzyme was challenged with substrates modified within the valine residue of the tripeptide. Initially this work was hampered by the relatively low levels of activity recoverable from the producing strain, a problem largely circumvented with the availability of larger quantities of protein, prepared using rDNA methods. The less-than-strict substrate specificity of the enzyme has been an important ingredient in this success, although the enzyme is not infallible as a catalyst, since one in approximately several hundred turnovers of substrate leads to irreversible inactivation,[9a] presumably due to an uncontrolled oxidative modification of the protein backbone.

The substrate analogues modified in this way that have been tested, are shown in Table 1, along with the characterized products of their turnover by the enzyme. Thus the analogue (7), containing D-α-aminobutyrate, is notable for the production[10] of both a penam (20) and a cepham (21). Subsequent to this work the closely related compounds (22) and (23) were reported[11] as naturally

[9a] D. Perry, E. P. Abraham, and J. E. Baldwin, *Biochem. J.*, 1988, **255**, 345.
[10] J. E. Baldwin, E. P. Abraham, R. M. Adlington, B. Chakravarti, A. E. Derome, J. A. Murphy, L. D. Field, N. B. Green, H. H. Ting, and J. Usher, *J. Chem. Soc., Chem. Commun.*, 1983, 1317.
[11] D. C. Aldridge, D. M. Carr, D. H. Davies, A. J. Hudson, R. D. Nolan, J. P. Poyser, and C. J. Strawson, *J. Chem. Soc., Chem. Commun.*, 1985, 1513.

Table 1 *Conversion of tripeptide analogues by IPN-synthase, modified in the valinyl residue*

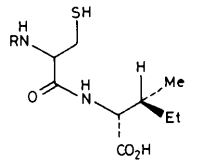
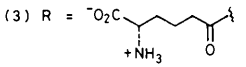
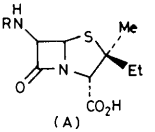
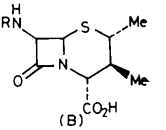
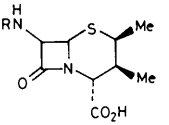
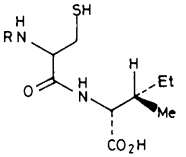
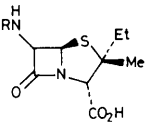
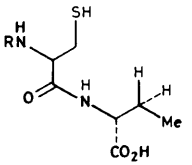
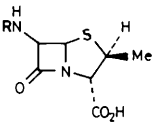
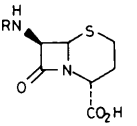
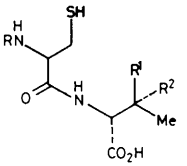
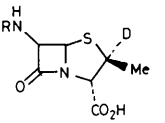
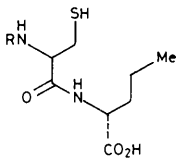
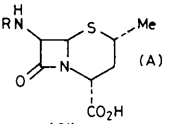
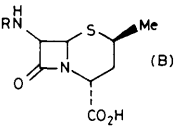
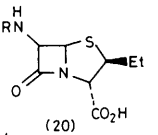
Substrate	*Products*	*Reference*
 (3) R = $^{-}O_2C$ 	 (A)  (B)  (C) A : B : C = 10 : 1 : 1	26—28
 (4)		26, 27
 (5)	 3 : 1 	10
 (6a) R^1 = H, R^2 = D (6b) R^1 = D, R^2 = H		27
 (7)	 (A) (21)  (B)  (20) A : B : C = 7 : 1 : trace	28

Table 1 *Continued*

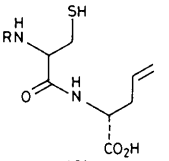
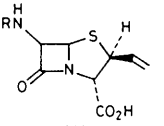
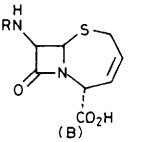
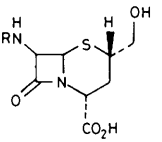
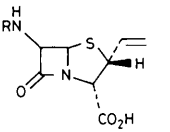
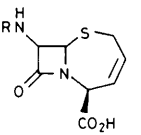
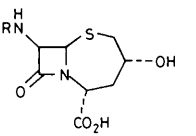
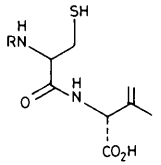
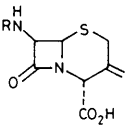
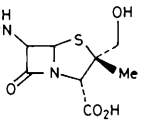
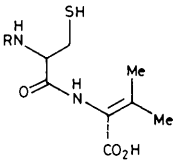
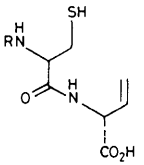
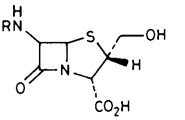
Substrate	*Products*	*Reference*
 (8)	 (A)  (B)  (C)  (D)  (E)  (F) A : B : C : D : E : F = 4 : 10 : 2 : 1 : 1 : 5	12, 29
 (9)	  3 : 1	12, 14
 (10)	no turnover	14
 (11)		14

Table 1 *Continued*

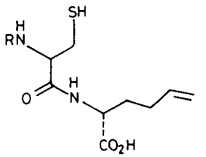
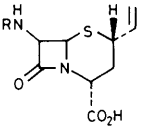
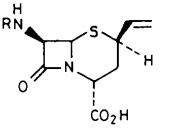
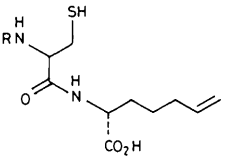
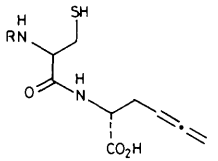
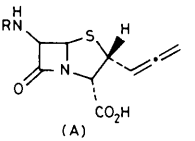
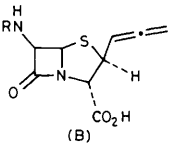
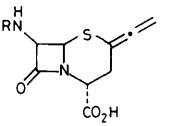
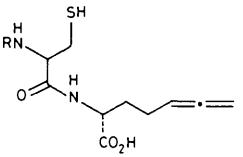
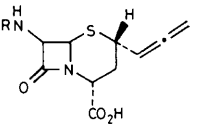
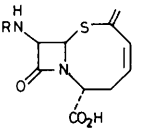
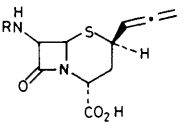
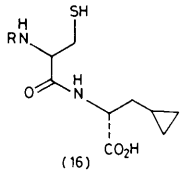
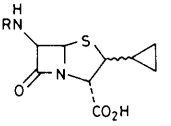
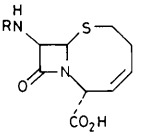
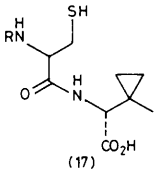
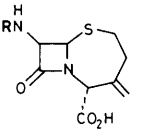
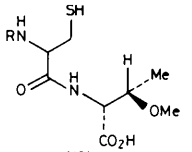
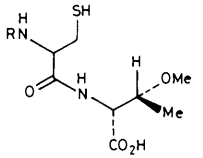
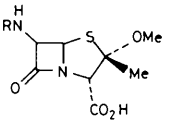
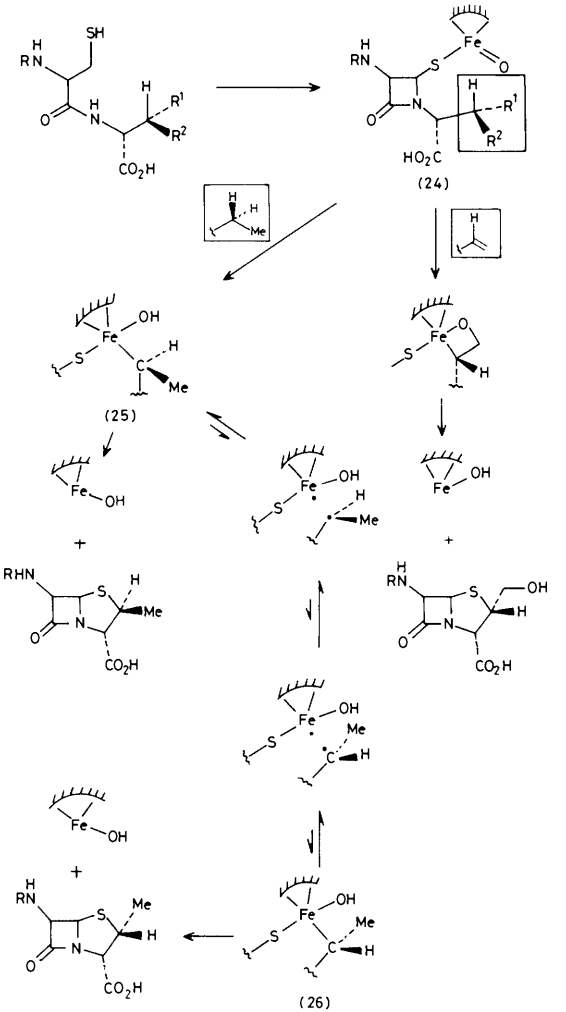
Substrate	*Products*	*Reference*
 (12)	 2 : 1 	14
 (13)	no turnover	14
 (14)	 (A)  (B)  (C) A : B : C = 20 : 10 : 7	15
 (15)	 (A)  (C)  (B) A : B : C = 3 : 1 : 7	15

Table 1 *Continued*

Substrate	*Products*	*Reference*
 (16)	  3 : 1	16
 (17)		16
 (18)	no conversion	30
 (19)		30

occurring constituents of a Streptomycete fermentation broth. Apparently, these natural products arise from an δ-(L-α-aminoadipyl)-L-cysteinyl-D-(α-aminobutyrate) intermediate, itself assembled from the constituent amino acids, perhaps on the same tripeptide synthetase involved in penicillin-N production in this organism. A unifying concept[12] that provides a compelling and satisfying rationalization of the observations collected in Table 1 is outlined in Scheme 3. Here the IPNS is depicted as an iron-dependent desaturase utilizing one dioxygen molecule per molecule of penicillin synthesized. Following irreversible closure of the β-lactam ring, one oxygen atom remains in the cyclization site to participate either in hydrogen atom abstraction (the normal catalytic path), or under favourable circumstances in the presence of unsaturation in the substrate, an oxygen atom can be donated to the substrate, leading to hydroxylation. The

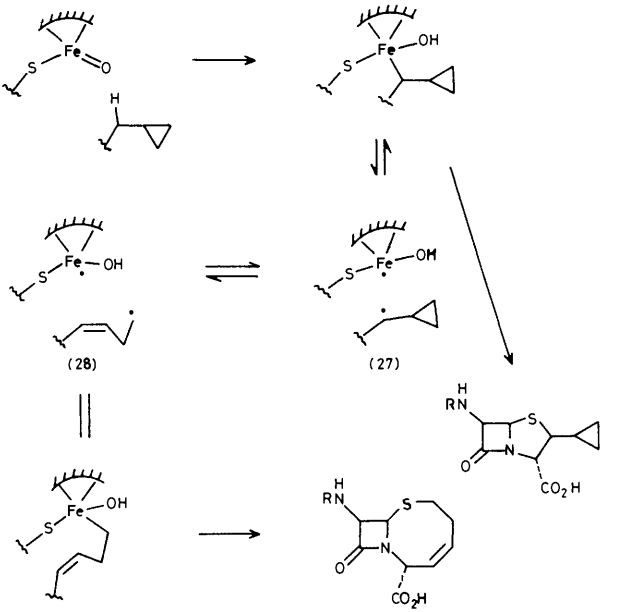
[12] J. E. Baldwin, R. M. Adlington, S. L. Flitsch, H. H. Ting, and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1986, 1305.



Scheme 3

iron-oxo species (24) has been suggested as a likely reactive intermediate capable of fulfilling both roles. As shown in Scheme 3, oxidative addition into a C–H bond might lead to species such as (25) or (26), and reductive elimination would then provide the new C–S bond. The plausible involvement of such radical species in this reaction finds some laboratory precedence,[13] and when derived from the analogues (6a) and (6b) these may become torsionally mobile, neatly explaining the observation of both retention [during turnover of (6a)] and

[13] J. E. Baldwin and T. S. Wan, *J. Chem. Soc., Chem. Commun.*, 1979, 249.

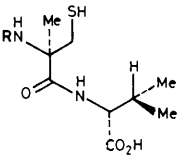
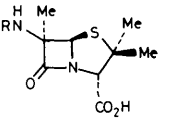
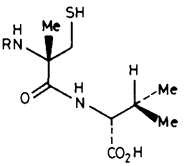
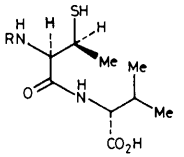
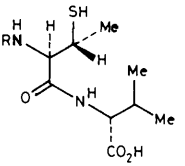
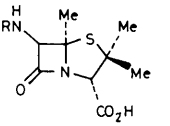


Scheme 4

inversion [during turnover of (6b)]; the overall stereochemical course will then be influenced by any topological bias imposed by the active site. In an alternative mode, a formal cycloaddition of the same iron-oxo species (24) to an olefin would lead to a metallocycle, and subsequently by reductive elimination, to a new C–S bond with concomitant hydroxylation of the substrate. However, product formation by the hydroxylation path depends critically upon the position of the alkene in the C-terminal residue of the tripeptide.[14] Hydroxylated products are only observed from the analogues (8), (9), and (11), whereas (12) gives only cepham products and not penams, and (10) and (13) are not turned over at all, within the limits of detection. Interestingly, the allene (14) with a double and in more or less the same relative location as that in (8) gave no products of hydroxylation, but instead only of desaturation.[15] More surprisingly, the cyclopropyl-containing substrates (16) and (17) are also turned over by the enzyme to give products arising not only from direct cyclization, but also ring expanded products *via* rearrangement of a presumptive cyclopropylcarbinyl radical (27) to a butenyl radical[16] (28) (Scheme 4). Apparently the enzyme is still able to maintain control over these reactive intermediates, to afford novel cyclized materials.

[14] J. E. Baldwin, R. M. Adlington, A. Basak, S. L. Flitsch, A. K. Forrest, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1986, 273.
[15] J. E. Baldwin, R. M. Adlington, A. Basak, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1986, 1280.
[16] J. E. Baldwin, R. M. Adlington, B. P. Domayne-Hayman, G. Knight, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1661.

Table 2 *Conversion of tripeptide analogues by IPN-synthase, modified in the cysteinyl residue*

Substrate	*Products*	*Reference*
 (29)		17
	no conversion	17
	no conversion	17
 (30)		17

Active site mapping of the IPNS has been pursued also with substrates modified in the cysteine and α-aminoadipyl residues. Although many valine substitutions provided substrates which were successfully cyclized to β-lactams, the enzyme places more stringent requirements upon the structural integrity of the cysteinyl moiety for cyclization to a β-lactam. Replacement of L-cysteine by L-serine, L-α-aminobutyrate or *S*-methyl-L-cysteine gave tripeptides which with IPNS were neither β-lactam-producing substrates, nor inhibitors of the normal reaction.[17] However, the successful conversion[17] of (2*R*)-2-methylcysteinyl and (2*R*,3*R*)-3-methylcysteinyl tripeptides [(29) and (30)] afforded the new β-lactams shown in Table 2. Apparently, *C*-methylation of the central cysteine residue can still give active substrates for IPNS, provided such modifications maintain a (2*R*) absolute configuration, and retain a 3-*pro*-*S* hydrogen, necessary for the

[17] J. E. Baldwin, R. M. Adlington, N. Moss, and N. G. Robinson, *J. Chem. Soc., Chem. Commun.*, 1987, 1664.

Table 3 *Tripeptide variants modified in the δ-(α-aminoadipoyl) moiety*

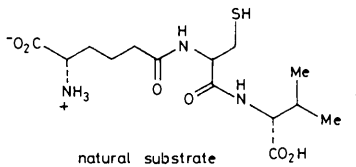
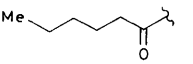
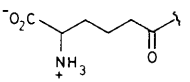
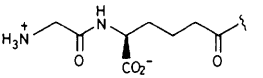
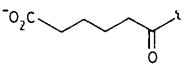
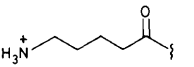
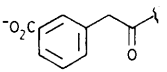
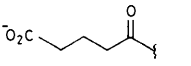
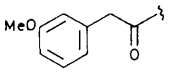
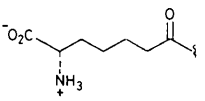
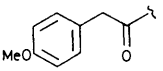
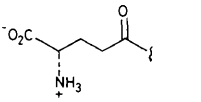
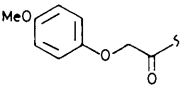
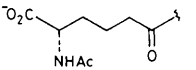
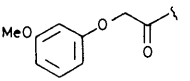
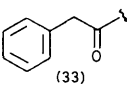
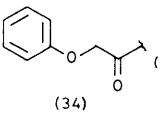
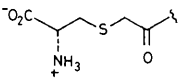
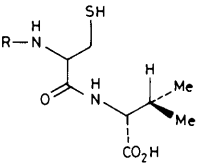
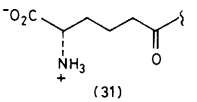
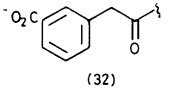
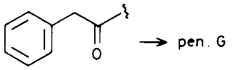
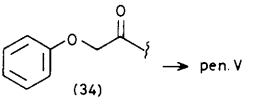
Substrates turned over by IPNS	*Ref.*	*Analogues not turned over*	*Ref.*
 natural substrate (31)	31		31
	31, 32		31
	31		31
 (32)	18, 33		31
	34		31
	34		31
	34		31
	34		
 (33)	19, 35—37 (slow turnover to pen. G)		
 (34)	19, 36 (slow turnover to pen. V)		
	31, 38, 39 (slow turnover)		

Table 4 *Kinetic parameters for the turnover of some tripeptides by isopenicillin-N synthase from* C. acremonium



Substrate (*R*)	*Reference*	K_m (mM)	$V_{max.}$ (μmol min^{-1})
 (31)	18	0.16	1.64
 (32)	18	0.8	0.8
 pen. G (33)	18	0.9	2.5×10^{-3}
 pen. V (34)	19	0.5	0.8×10^{-3}

formation of an enzyme-bound monocyclic β-lactam in the first stage of the transformation.

Substrate analogues bearing changes in the α-aminoadipyl residue, tested with the enzyme, are shown in Table 3. The assays have been performed with purified IPNS from *C. acremonium*, as well as with extracts from *P. chrysogenum* and *S. clavuligerus* which contain IPNS activity. With this in mind, these results show as a minimum structural requirement for conversion into penicillin products, that the *N*-acyl group should have a six carbon or equivalent chain, terminating in a carboxyl group. An interesting comparison[18,19] has been made of the steady-state kinetics for turnover of tripeptides (31), (32), and (33) (see Table 4).

[18] J. E. Baldwin, R. M. Adlington, M. J. C. Crabbe, G. C. Knight, T. Nomoto, and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1987, 806.

[19] J. E. Baldwin, E. P. Abraham, G. L. Burge, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1985, 1808.

Although the substrates (32) and (33) have very similar K_m values, the $V_{max.}$ for (32) is much larger than for (33), and is closer to the $V_{max.}$ observed with the natural substrate (31), implying that once bound the *meta*-carboxy function of (32) (and hence the terminal carboxyl in LLD-ACV), helps to orient the tripeptide into an optimal fit with the protein for ring closure at the catalytic site.[18]

These studies illustrate well the potential of the IPNS as a catalyst for the production of novel β-lactam structures. It is conceivable that by altering the structure of the protein by mutagenesis, the specificity and catalytic efficiency of the enzyme might be tailored, or improved, for the production of yet other new β-lactams, or products such as penicillin-V and penicillin-G *directly* from an appropriate tripeptide precursor [*i.e.* (34) and (33)].

The full primary sequence of the IPNS from *C. acremonium*,[20] *P. chrysogenum*,[21] and *Aspergillus nidulans*[22] have now been derived from their corresponding encoding nucleotide sequences. The protein from *C. acremonium* was the first to be cloned and expressed in *E. coli*. The IPNS gene was identified by purifying the protein, determining the N-terminal sequence of 23 amino acids, preparing a set of synthetic oligonucleotides encoding a portion of this sequence, and probing a cosmid genomic library with the mixed oligonucleotide probe. The DNA sequence of a portion of one cosmid clone was then seen to match the experimentally determined amino acid sequence, although the IPNS open reading frame begins with methionine and glycine, which are not found in the protein isolated from *C. acremonium*. These residues are apparently cleaved post-translationally. This open reading frame, in an *E. coli* expression system, affords *E. coli* cells producing about 20% of total cell protein as the IPNS polypeptide. This recombinant IPNS, whilst undergoing slightly different N-terminal processing has unaltered steady-state kinetics for the conversion of LLD-ACV into isopenicillin-N.[23] Following this work, the IPNS gene from *P. chrysogenum* was isolated from a recombinant λ-library using the *C. acremonium* IPNS gene as a heterologous hybridization probe.[21] After DNA sequencing, the protein coding region of the two genes were seen to be about 74% homologous, and the predicted amino acid sequences were 73% homologous. This high homology is not surprising given that both genes are isolated from fungal sources, and they may well have evolved from a common ancestral gene. The origins of this putative ancestral gene may have been the Gram positive bacterial genus *Streptomyces*. It is of interest, therefore, that the IPNS gene from *S. clavuligerus* was isolated from an *E. coli* plasmid library recently,[24] and sequenced. The protein coding region of the *S. clavuligerus* IPNS gene shows about 63% and

[20] S. M. Samson, R. Belagaje, D. T. Blankenship, J. L. Chapman, D. Perry, P. L. Skatrud, R. M. VanFrank, E. P. Abraham, J. E. Baldwin, S. W. Queener, and T. D. Ingolia, *Nature*, 1985, **318**, 191.
[21] L. G. Carr, P. L. Skatrud, M. E. Scheetz, S. W. Queener, and T. D. Ingolia, *Gene*, 1986, **48**, 257.
[22] D. Raman, L. Carramolino, C. Patino, F. Sanchez, and M. A. Penalva, *Gene*, 1987, **57**, 171.
[23] J. E. Baldwin, S. J. Killin, A. J. Pratt, J. D. Sutherland, N. J. Turner, M. J. C. Crabbe, E. P. Abraham, and A. C. Willis, *J. Antibiot.*, 1987, **40**, 652.
[24] B. K. Leskiw, Y. Aharonowitz, M. Mezarech, S. Wolfe, L. C. Vining, D. W. S. Westlake and S. E. Jensen, *Gene*, 1988, **62**, 187.

Table 5 *Specific activity of wild-type and mutant IPNS proteins (ref. 25)*

Protein	*Specific activity* (units/mg)	K_m (mM)
Wild-type	0.59	0.15
Ser-106 mutant	0.03	0.70
Ser-255 mutant	0.29	0.15
Ser-106, Ser-255 mutant	0.02	0.77

62% similarity to the *C. acremonium* and *P. chrysogenum* IPNS coding sequences, respectively, and the predicted amino acid sequence shows about 56% similarity to both fungal sequences, confirming the close evolutionary relationship between the fungal and bacterial enzymes. The *S. clavuligerus* open reading frame encodes a protein of M_r 36 917. Finally, the IPNS gene from *Aspergillus nidulans* has also been cloned,[22] again using the *C. acremonium* gene as a hybridization probe.

The high homology apparent in the IPNS sequences to some extent hampers attempts to assign functional roles to conserved regions of the protein; it is clearly not only the catalytically important residues that are conserved. There are twelve separate regions where the three fungal proteins match for at least eight consecutive amino acid residues, whereas there are only three such extensive regions of similarity with the *S. clavuligerus* protein. In addition, the three fungal enzymes each contain two cysteine residues (Cys-106, Cys-255 in *C. acremonium* numbering), nested in conserved regions of the protein, whilst the prokaryotic protein has four cysteines. The positions of two of the cysteine residues in the *S. clavuligerus* sequence are analogous to those in the fungal proteins. And in each case, within 5—10 residues downstream of these cysteines is also located a histidine residue. It is likely that one or more of these amino acids provides ligands for the catalytically essential Fe^{2+} atom. Recently three mutant cIPNS proteins have been prepared by site-directed mutagenesis.[25] These carry con-

[25] S. M. Samson, J. L. Chapman, R. Belagaje, S. W. Queener, and T. D. Ingolia, *Proc. Nat. Acad. Sci. USA.*, 1987, **84**, 5705.
[26] G. A. Bahadur, J. E. Baldwin, J. J. Usher, E. P. Abraham, J. S. Jayatilake, and R. L. White, *J. Am. Chem. Soc.*, 1981, **103**, 7650.
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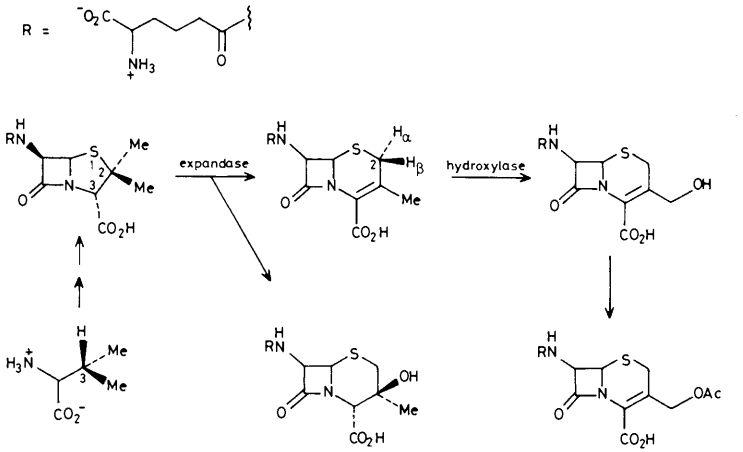
[28] J. E. Baldwin, R. M. Adlington, N. J. Turner, B. P. Domayne-Hayman, H. H. Ting, A. E. Derome, and J. A. Murphy, *J. Chem. Soc., Chem. Commun.*, 1984, 1167.
[29] J. E. Baldwin, R. M. Adlington, A. E. Derome, H. H. Ting, and N. J. Turner, *J. Chem. Soc., Chem. Commun.*, 1984, 1211.
[30] J. E. Baldwin, R. M. Adlington, A. Basak, S. L. Flitsch, S. Petursson, N. J. Turner, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1986, 975.
[31] J. E. Baldwin, E. P. Abraham, R. M. Adlington, G. A. Bahadur, B. Chakravarti, B. P. Domayne-Hayman, L. D. Field, S. L. Flitsch, G. S. Jayatilake, A. Spakovskis, H. H. Ting, N. J. Turner, R. L. White, and J. J. Usher, *J. Chem. Soc., Chem. Commun.*, 1984, 1225.
[32] A. L. Demain, Y. Q. Shen, S. E. Jensen, D. W. Westlake, and S. Wolfe, *J. Antibiot.*, 1986, **39**, 1007.
[33] J. E. Baldwin, R. M. Adlington, M. J. C. Crabbe, T. Nomoto, and C. J. Schofield, *Tetrahedron*, 1987, **43**, 4217.
[34] J. E. Baldwin, A. J. Pratt, and M. G. Moloney, *Tetrahedron*, 1987, **43**, 2565.
[35] J. M. Luengo, M. T. Alemay, F. Salto, F. Ramos, M. J. Lopez-Nieto, and J. F. Martin, *Biotechnology*, 1986, **4**, 44.
[36] S. E. Jensen, D. W. S. Westlake, R. J. Bowers, L. Lyubechansky, and S. Wolfe, *J. Antibiot.*, 1986, **39**, 822.

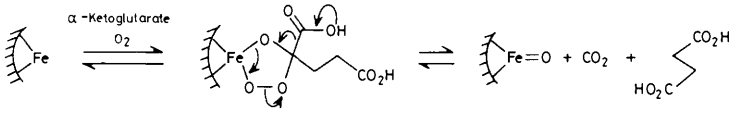


Scheme 5

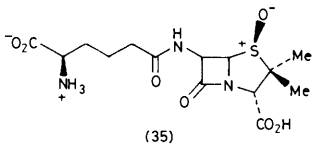
servative substitutions of the cysteines 106 and 255, for serine, and the kinetic parameters for these proteins are given in Table 5. The most notable feature is that all three retain some enzymatic activity, showing that the replacement –OH groups are at least partly able to fulfil the functional role of the thiols in the catalytic cycle. It seems unlikely, therefore, that these thiols participate in intramolecular disulphide bond formation, or in the formation of a disulphide bond with the substrate. Whilst a precise interpretation of these results is difficult, a more intimate role for Cys-106 in the chemistry is indicated by the higher K_m, and the much reduced specific activity of the Ser-106 mutant protein.

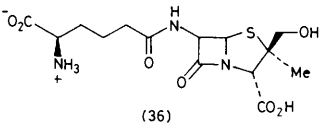
B. Deacetoxycephalosporin-C Synthetase/Hydroxylase.—Following the conversion of isopenicillin-N into penicillin-N, a ring-expansion reaction leads us away from the penam skeleton, and introduces the cephalosporin system (Scheme 1). The enzymic ring-expansion of penicillin-N to deacetoxycephalosporin-C (DAOC), and the subsequent hydroxylation to afford deacetylcephalosporin-C (DAC) (Scheme 5), by a cell-free extract from *C. acremonium* was first detected in 1976,[40] and was established rigorously by 1980.[41,42] Both the DAOC synthetase and DAC hydroxylase activities were shown to require the addition of Fe^{2+}, ascorbate, and α-ketoglutarate for

[37] J. M. Luengo, M. J. Lopezenie, and F. Salto, *J. Antibiot.*, 1986, **39**, 1144.
[38] R. J. Bowers, S. E. Jensen, L. Lyubechansky, D. W. S. Westlake, and S. Wolfe, *Biochem. Biophys. Res. Commun.*, 1984, **120**, 607.
[39] J. E. Shields, C. S. Campbell, S. W. Queener, D. C. Duckworth, and N. Nuess, *Helv. Chim. Acta*, 1984, **67**, 870.
[40] M. Kohsaka and A. L. Demain, *Biochem. Biophys. Res. Commun.*, 1976, **70**, 465.
[41] J. E. Baldwin, P. D. Singh, M. Yoshida, Y. Sawada, and A. L. Demain, *Biochem. J.*, 1980, **186**, 889.
[42] J. E. Baldwin, J. W. Keeping, P. D. Singh, and C. A. Vallejo, *Biochem. J.*, 1981, **194**, 649.



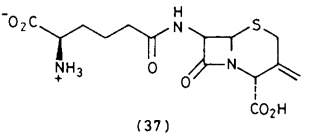
Scheme 6



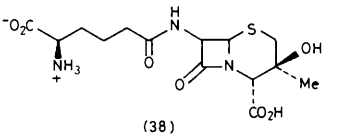


maximum activity.[43–47] At the same time, extracts of *Streptomyces clavuligerus*, which produces a number of β-lactam compounds including penicillin-N, cephamycin-C, and clavulanic acid, were shown,[47,48] to contain DAOC synthetase and DAC hydroxylase activities with the same cofactor requirements. Later, the partial purification and separation of these activities, from this prokaryote, were achieved,[49] with molecular weight estimates of 29 500 and 26 200 kDa for the synthetase and hydroxylase, respectively. However, it was clear already that the DAOC synthetase and DAC hydroxylase activities from the fungal source *C. acremonium* were inseparable and resided on a single bifunctional enzyme.[50] Recently the purification[51,52] and cloning[53] of a single polypeptide of M_r 40 000 from *C. acremonium* has been reported, which possess both the 'expandase' and hydroxylase activities. Crucial to this success was the ability to assay expandase and hydroxylase activities at very low levels, using a sensitive bioassay against a supersensitive *E. coli*, and a t.l.c./h.p.l.c.-based bioconversion assay.[54] This bifunctional enzyme requires α-ketoglutarate, Fe^{2+}, and O_2 as cofactors, and ascorbate and dithiothreitol were also necessary for maximal activity. Both sequential steps consume dioxygen and α-ketoglutarate, and the latter is degraded into succinate and CO_2 (Scheme 6). The enzyme acts as a true dioxygenase in the hydroxylation step, by inserting one atom of oxygen into the

[43] D. J. Hook, L. T. Chang, R. P. Elander, and R. B. Morin, *Biochem. Biophys. Res. Commun.*, 1979, **876**, 258.
[44] S. J. Brewer, J. E. Farthing, and M. K. Turner, *Biochem. Soc. Trans.*, 1977, **5**, 1024.
[45] H. R. Felix, J. Nuesch, and W. Wehrli, *FEMS Microbiol. Lett.*, 1980, **8**, 55.
[46] J. Kupka, Y. Q. Shen, S. Wolfe, and A. L. Demain, *FEMS Microbiol. Lett.*, 1983, **16**, 1.
[47] M. K. Turner, J. E. Farthing, and S. J. Brewer, *Biochem. J.*, 1978, **173**, 839.
[48] S. E. Jensen, D. W. S. Westlake, R. J. Bowers, and S. Wolfe, *J. Antibiot.*, 1982, **35**, 1351.
[49] S. E. Jensen, D. W. S. Westlake, and S. Wolfe, *J. Antibiot.*, 1985, **38**, 263.
[50] A. Scheidegger, M. T. Kuenzi, and J. Nuesch, *J. Antibiot.*, 1984, **37**, 522.
[51] J. E. Baldwin, R. M. Adlington, J. B. Coates, M. J. C. Crabbe, N. P. Crouch, J. W. Keeping, G. C. Knight, C. J. Schofield, H. H. Ting, C. A. Vallejo, M. Thorniley, and E. P. Abraham, *Biochem. J.*, 1987, **245**, 831.
[52] J. E. Dotzlaf and W. K. Yeh, *J. Bacteriol.*, 1987, **169**, 1611.
[53] S. M. Samson, J. E. Dotzlaf, M. L. Slisz, G. W. Becker, R. M. VanFrank, L. E. Veal, W. Kuang Yeh, R. J. Miller, S. W. Queener, and T. D. Ingolia, *Biotechnology*, 1987, **5**, 1207.
[54] J. E. Baldwin and M. J. C. Crabbe, *FEBS Lett.*, 1987, **214**, 357.



(37)



(38)

substrate,[55] and the second into the cofactor α-ketoglutarate during its oxidative decarboxylation to succinate and CO_2. No enzyme-free intermediates have so far been discovered during these conversions.

By analogy with known non-enzymic conversions of penicillins into cephalosporins, the β-sulphoxide (35) and the β-hydroxymethyl penam (36) have been proposed as feasible intermediates in the expandase reaction, but neither was converted into cephalosporins when incubated with the purified enzyme.[51] The enzymic reaction occurs with loss of two hydrogen atoms from the substrate, one from the 2-*pro-R* methyl group,[1] and the other from the C-3 position (Scheme 5). Feeding experiments in a whole-cell system with L-valine bearing a chiral methyl group at the 3-*pro-R* site, gave cephalosporin-C with equal tritium content at the C-2α and C-2β sites.[56,57] Given a normal primary kinetic isotope effect on the C–H bond cleavage,[58] this indicates a loss of stereocontrol during formation of the new C–S bond. On the other hand, the allylic oxidation of DAOC carrying a chiral C-3 -methyl group occurs with retention of configuration.[59] Thus the protein catalyses two types of chemical reaction, the ring expansion occurring with loss of stereocontrol, and the hydroxylation proceeding stereospecifically. These activities are reminiscent of the IPNS reaction, which can also promote hydroxylation events when challenged with substrates containing double bonds. Moreover the *C. acremonium* DAOC/DAC synthetase will also convert the methylene cephalosporin-C (37) into deacetylcephalosporin,[60] with the added oxygen being derived from molecular oxygen.[55] The closely related 3β-hydroxycepham (38) was isolated[61] in 1981 from the filtered fermentation broth of *C. acremonium*, whereas more recently it was observed that the incubation of penicillin-N with the fungal enzyme also gave in minor amounts this same compound,[62] in addition to DAOC and DAC (Scheme 5). However, on repeating the incubation with [3-^{2}H]penicillin-N the same three products were seen, with a far greater proportion of (38) now

[55] J. E. Baldwin, R. M. Adlington, C. J. Schofield, N. P. Crouch, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1556.
[56] C. P. Pang, R. L. White, E. P. Abraham, D. H. G. Crout, M. Lutsorf, P. J. Morgan, and A. E. Derome, *Biochem. J.*, 1984, **222**, 777.
[57] C. A. Townsend, A. B. Theis, A. S. Neese, E. B. Barrabee, and D. Polund, *J. Am. Chem. Soc.*, 1985, **107**, 4760.
[58] J. E. Baldwin, R. M. Adlington, R. T. Alpin, N. P. Crouch, C. J. Schofield, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1654.
[59] C. A. Townsend and E. B. Barrabee, *J. Chem. Soc., Chem. Commun.*, 1984, 1586.
[60] R. M. Adlington, J. E. Baldwin, B. Chakravarti, M. Jung, S. E. Moroney, J. A. Murphy, P. D. Singh, J. J. Usher, and C. Vallejo, *J. Chem. Soc., Chem. Commun.*, 1983, 153.
[61] R. D. Miller, L. L. Huckstep, J. P. Mcdermott, S. W. Queener, S. Kukolja, D. O. Spry, T. K. Elzay, S. M. Lawrence, and N. Nuess, *J. Antibiot.*, 1981, **34**, 984.

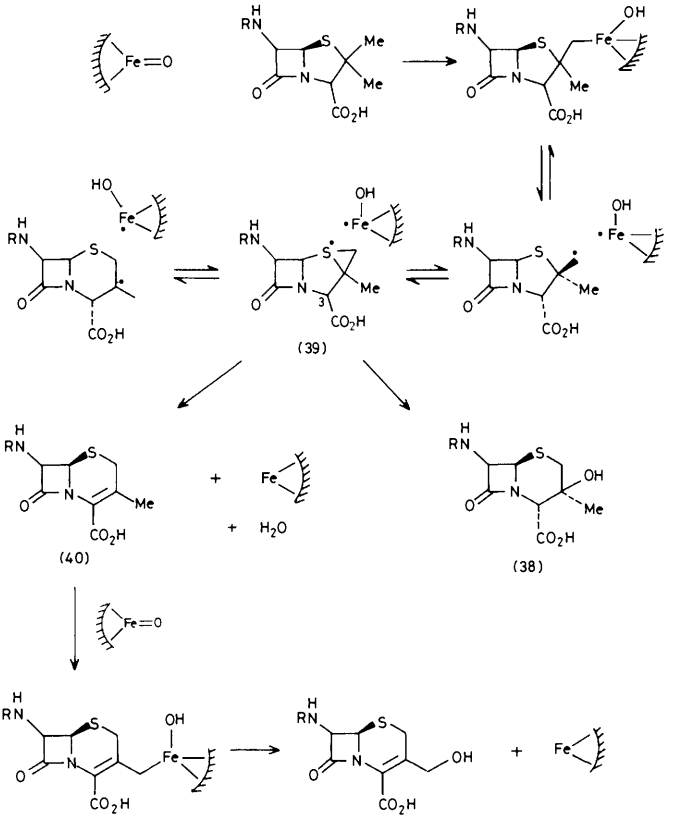
present, and the 3β-hydroxyl oxygen being derived from molecular oxygen.[62] This indicates the possibility of a branched pathway in the catalytic cycle leading to DAOC; thus an intermediate (39; Scheme 7)[63] may collapse to give either (38) or (40), and the ratio of these events is sensitive to the presence of ^2H at C-3. That the conversion of penicillin-N into DAOC is a stepwise process was also shown recently[63] in competitive mixed-label turnover experiments. Thus reaction of a 1:1 mixture of penicillin-N and 2,2-bis(trideuteriomethyl)penicillin-N resulted in preferential conversion of the protio substrate, whereas in similar experiments with a 1:1 mixture of penicillin-N and 3-deuteriopenicillin-N, both substrates were converted at the same rate. This is in accord with a stepwise process in which the methyl hydrogen is removed first, followed in a later step by the methine hydrogen. The mechanism proposed[63] to account for these observations is shown in Scheme 7. It is not yet clear whether or not the second oxidation (*i.e.* hydroxylation) occurs in the same active site as does the first, although this might appear unlikely. The involvement of radical intermediates in the ring expansion finds some precedence in the biomimetic process[64] depicted in Scheme 8.

The gene coding for this bifunctional DAOC/DAC synthetase was cloned recently[53] using oligonucleotide probes based on amino acid sequences of peptide fragments derived from purified protein. Direct amino acid sequencing of the protein was unsuccessful, due apparently to N-terminal blockage *in vivo*. When sequenced the gene revealed a single open reading frame which encoded a protein of M_r 36 462. Again the open reading frame could be expressed in *E. coli* and the DAOCS and DACS activities were found in a 9:1 ratio, which is about the same as that observed[52] for the bifunctional enzyme purified from *C. acremonium*. The purified polypeptide from *E. coli* was indistinguishable from the 'natural' enzyme, both by SDS-PAGE and by tryptic cleavage patterns.[53] Notable in the primary sequence is the presence of five cysteine residues, and one, Cys-100, occurs in a ten-residue sequence showing 50% homology to the region containing the Cys-106 residue in IPNS, suggesting that this Cys-100 in DAOC/DAC synthetase fulfils a similar role to that of the Cys-106 in IPNS.

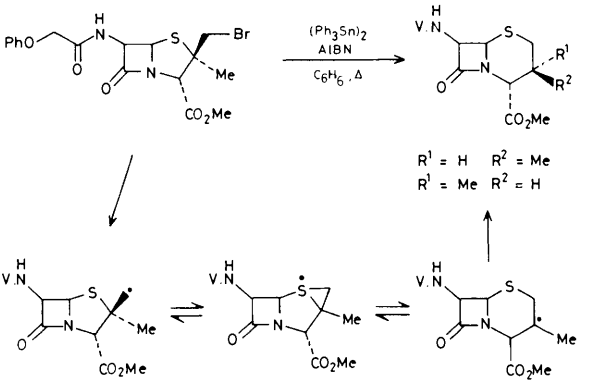
The specificity of the enzyme has been probed[65,66] by challenging the enzyme with substrate analogues modified in the acylamino side chain of penicillin-N. Apparently a six-carbon chain terminating in a carboxyl group permits efficient conversion into cephams, with the exception[50,67] of the δ(L-α-aminoadipoyl) side chain, *i.e.* isopenicillin-N (see Table 6).

Returning to the question as to whether a single active site or two active sites

[62] J. E. Baldwin, R. M. Adlington, R. T. Aplin, N. P. Crouch, G. Knight, and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1987, 1651.
[63] J. E. Baldwin, R. M. Adlington, R. T. Alpin, N. P. Crouch, C. J. Schofield, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 1654.
[64] J. E. Baldwin, R. M. Adlington, T. W. Kang, E. Lee, and C. J. Schofield, *J. Chem. Soc., Chem. Commun.*, 1987, 104.
[65] J. E. Baldwin, R. M. Adlington, J. B. Coates, M. J. C. Crabbe, J. W. Keeping, G. C. Knight, T. Nomoto, C. J. Schofield, and H. H. Ting, *J. Chem. Soc., Chem. Commun.*, 1987, 374.
[66] J. E. Baldwin, *et al.*, *Tetrahedron*, 1987, **43**, 3009.
[67] J. Kupka, Y.-Q. Shen, S. Wolfe, and A. L. Demain, *Can. J. Microbiol.*, 1983, **29**, 488.

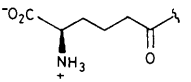
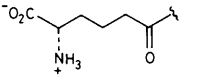
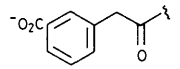
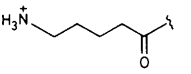
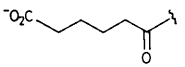
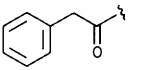
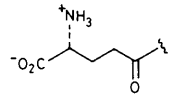
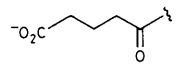
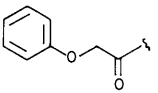


Scheme 7



Scheme 8

Table 6 *Substrates converted by expandase, and analogues that do not act as substrates*

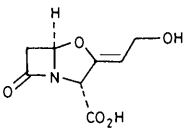
Substrate	*Relative rate of turnover*	*No conversion*
	100	
	50	
	40	
	14	
	3	

(distinct or overlapping) are responsible for the dual DAOC/DAC synthase activities of the *C. acremonium* enzyme, evidence was mentioned earlier for separable expandase and hydroxylase activities in extracts of *Streptomyces clavuligerus*, suggesting that here the enzymes are coded on separate polypeptides. The *C. acremonium* polypeptide may well have evolved by fusion of contiguous but separate reading frames encoding the two separate activities in the prokaryotic organism, but it is not yet clear how these are organized in *S. clavuligerus*. It will certainly be of interest to determine whether separate contiguous genes for expandase and hydroxylase occur in Streptomycetes, and to compare their protein coding regions with that already known for the eukaryotic bifunctional protein.

C. Clavaminic Acid Synthase.—Clavulanic acid (41) is a commercially valuable β-lactamase inhibitor, produced by several strains of Streptomyces, in particular *S. clavuligerus*. Biosynthetic studies led initially to the identification of putative primary precursors, and have culminated recently in the detection of several advanced intermediates, the cloning of a genetic locus involved in clavulanic acid production in *S. clavuligerus*, and the purification of a novel Fe^{2+}, O_2, and α-ketoglutarate-linked dioxygenase responsible for closure of the oxazolidene ring.

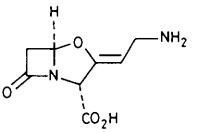
Clavulanic acid is assembled from C_3 and C_5 precursors, whose identity has so far been sought using whole-cell studies. Thus labelled glycerol,[68] glyceric acid,[69]

[68] S. W. Elson and R. S. Oliver, *J. Antibiot.*, 1978, **31**, 586.
[69] C. A. Townsend and M. F. Ho, *J. Am. Chem. Soc.*, 1985, **107**, 1066.

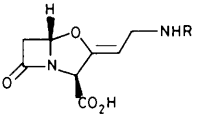


clavulanic acid

(41)



(46)



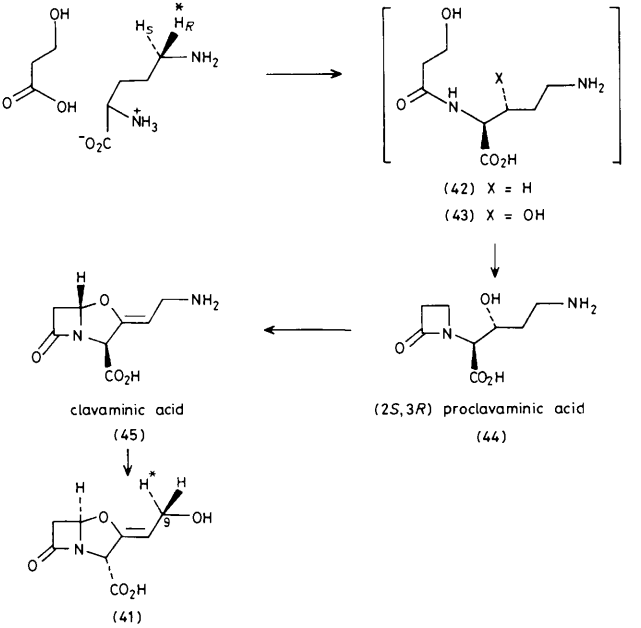
(47) R = COMe

(48) R = CO·CH_2NH_2

(49) R = CO·$CH_2NHCOMe$

and β-hydroxypropionic acid[70] each act as efficient precursors of the C_3 β-lactam section (Scheme 9), whereas glutamate[71] and ornithine[72,73] may supply the C_5 moiety. This knowledge, however, has been sufficient to establish important stereochemical features of the assembly process. Thus the incorporations[72] of (2*RS*,5*R*) and (2*RS*,5*S*)[5-^{3}H]ornithine into clavulanic acid occurred with retention of the 5-*pro*-*R* hydrogen, loss of the 5-*pro*-*S* hydrogen. The former subsequently assumes the 9-*pro*-*S* position in clavulanate, with overall inversion of configuration, possibly following the action of a transaminase or an amine oxidase, and an alcohol dehydrogenase. On the other hand, evidence concerning the overall stereochemical course of β-lactam ring formation was obtained,[74] from the incorporations of (1*R*,2*R*) and (1*S*,2*R*)[1-^{3}H][1,3-^{14}C]glycerol; the retention of ^{3}H label only from the *R*-isomer indicates overall retention during β-lactam ring closure, assuming that the steps linking glycerol and the actual biosynthetic precursor (possibly β-hydroxypropionate) do not also affect this centre. In this respect a notable similarity exists to β-lactam formation in the penicillin series, which also occurs with retention at C-3 in the cysteine residue. Extending the analogy further leads to a hypothetical dipeptide (42), or to the analogous dipeptide (43) containing β-hydroxyornithine, as possible early intermediates,[70] although such species have not been detected in fermentations of *S. clavuligerus*, nor tested as biosynthetic precursors. However, recent studies[75] have led to the isolation from cell extracts of two intermediates on this pathway which possess the structures (44) and (45), where remarkably, the absolute configuration of (45) is opposite to that of clavulanic acid, as judged by comparison of CD spectra of the natural metabolite and synthetic 9-amino-deoxyclavulanate (46) produced chemically from clavulanic acid. The absolute configuration of the biologically active enantiomer of proclavaminic acid (44) is (2*S*,3*R*), as deduced through its total synthesis.[76,77] The direct precursor role of

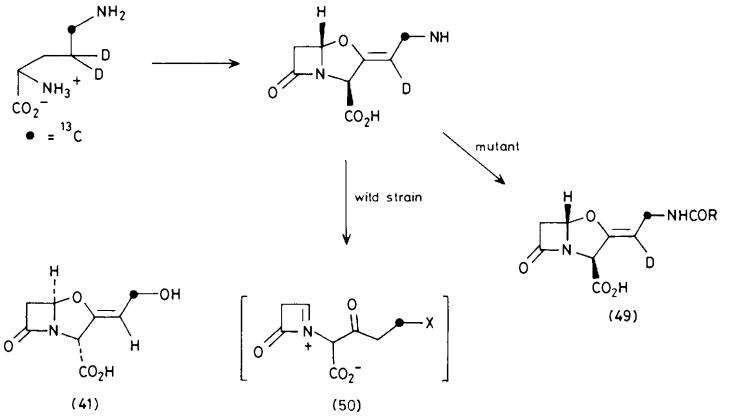
[70] A. L. Gutman, V. Ribon, and A. Boltanski, *J. Chem. Soc., Chem. Commun.*, 1985, 1627.
[71] S. W. Elson, R. S. Oliver, B. W. Bycroft, and E. A. Farouk, *J. Antibiot.*, 1982, **35**, 81.
[72] C. A. Townsend, M. F. Ho, and S. S. Mao, *J. Chem. Soc., Chem. Commun.*, 1986, 638.
[73] B. W. Bycroft, A. Penrose, J. Gillett, and S. W. Elsen, *J. Chem. Soc., Chem. Commun.*, 1988, 980.
[74] C. A. Townsend and S. Mao, *J. Chem. Soc., Chem. Commun.*, 1987, 86.
[75] S. W. Elson, K. H. Baggaley, J. Gillett, S. Holland, N. H. Nicholson, J. T. Sime, and S. R. Waroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1736.
[76] K. H. Baggaley, N. H. Nicholson, and J. T. Sime, *J. Chem. Soc., Chem. Commun.*, 1988, 567.
[77] K. H. Baggaley, J. T. Sime, N. H. Nicholson, S. W. Elson, J. Gillett, S. Holland, and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1738.



Scheme 9

proclavaminic acid (44) in clavulanate biosynthesis was demonstrated[78] by the synthesis and incorporation of ^{13}C-labelled material using a whole-cell feeding experiment, whereas the cell-free conversion of clavaminic acid into clavulanic acid in the presence of pyridoxal phosphate, pyruvate, and NADPH, could only be observed[78] in low yield (0.3%). Three *N*-acyl derivatives of clavaminic acid, (47), (48), and (49), have also been isolated[79] from a mutant of *S. clavuligerus* blocked in the clavulanic acid pathway between (45) and (41), suggesting that in this organism, (45) accumulates intracellularly, is acylated and then excreted into the medium. Most interestingly, an enzyme present in cell-free extracts, which cyclizes (44) to give (45), has been purified to homogeneity.[75] The enzyme shows M_r 49 200 by SDS-PAGE, and 47 000 by PAGE under non-denaturing conditions, it requires Fe^{2+}, α-ketoglutarate, and O_2 as cofactors/co-substrates, and consumes two equivalents of α-ketoglutarate for each equivalent of clavaminic acid formed. The enzyme has been named clavaminic acid synthetase.[75] Its mechanism of action is so far unknown, but in catalysing the removal of four hydrogens from the substrate, along with ring closure and enol ether formation, it has clear similarities to the Fe^{2+}/α-ketoglutarate-linked dioxygenases operating

[78] S. W. Elson, K. H. Baggaley, J. Gillet, S. Holland, N. H. Nicholson, J. T. Sime, and S. R. Woroniecki, *J. Chem. Soc., Chem. Commun.*, 1987, 1739.
[79] S. W. Elson, J. Gillett, N. H. Nicholson, and J. W. Tyler, *J. Chem. Soc., Chem. Commun.*, 1988, 979.

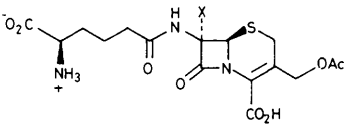


Scheme 10

in the penicillin/cephalosporin pathway. There is also the intriguing stereochemical relationship between clavaminic acid and clavulanic acid; it has not yet been explained why such an advanced intermediate should have the opposite absolute configuration to the final product, nor by what mechanism this is corrected as clavaminic acid is converted into clavulanic acid. Relevant to this latter point, the incorporation of [4-$^{2}H_2$, 5-^{13}C]ornithine into (49) was seen[80] to occur specifically with enrichments of ^{13}C at C-9 and ^{2}H at C-8 (Scheme 10), whereas the same labelled precursor was incorporated into (41) with enrichment of ^{13}C at C-9, but with the complete loss of deuterium at C-8. This observation supports but does not prove the involvement of an intermediate such as (50) in the conversion of (45) into (41).

In complementary studies, genes for some of the biosynthetic enzymes on this pathway have been cloned from *S. clavuligerus*. This has been achieved[81] using the high copy *Streptomyces* plasmid plJ 702, into which were inserted Bgl II chromosomal fragments. This pool of recombinant plasmids was then used to transform an *S. clavuligerus* mutant which makes normal levels of the other antibiotics produced by the strain, but undetectable levels of clavulanic acid. The transformants were screened for the restored ability to inhibit β-lactamase, and of the 5 000 screened two positives were identified. The plasmid DNA from these two transformants were shown to contain inserts spanning 23 kb of chromosomal DNA, with a common overlapping sequence of about 2 kb. It is likely that this chromosomal DNA encodes most if not all of the biosynthetic genes, although the location and organization of specific genes in the cluster remains to be established.

[80] B. W. Bycroft, A. Penrose, J. Gillett, and S. W. Elson, *J. Chem. Soc., Chem. Commun.*, 1988, 980.
[81] C. R. Bailey, M. J. Butler, I. D. Normansell, R. T. Rowlands, and D. J. Winstanley, *Biotechnology*, 1984, **2**, 808.



(51) X = OMe
(52) X = H

D. Other Enzymes in β-Lactam Biosynthesis.—Progress has been made in the purification of other enzymes acting in penicillin and cephalosporin biosynthesis, and this work is described in a recent review.[82] Notably, an isopenicillin-N acylase has been characterized from *P. chrysogenum*, which catalyses the formation of 6-aminopenicillanic acid from both isopenicillin-N and benzylpenicillin. A transacylase catalysing the transfer of a phenylacetyl group from phenylacetyl-CoA to 6-APA has also been detected.

The formation of isopenicillin-N requires first the construction of the L,L,D-ACV tripeptide. As yet little is known of the enzymology surrounding the formation of the tripeptide from the constituent amino acids. The assembly is not ribosomally mediated, and the precursor L-valine undergoes a stereochemical inversion at the C-α centre during its insertion into the peptide.[83] The failure to obtain cell-free extracts with more than a very low activity appears to have hindered further studies of the enzymes involved, although this situation may change once the genes encoding these proteins have been identified.

The conversion of isopenicillin-N into penicillin-N is an essential part of the branch leading to the cephalosporins. An epimerase has been detected[84] and partially purified[85,86] that catalyses this conversion. The molecular weight was estimated to be about 60 000, and although unstable, the activity is protected by pyridoxal phosphate, suggesting a role for this coenzyme in the reaction.

The 7-α-methoxycephalosporin-C (51) is also produced by *S. clavuligerus*, from cephalosporin-C (52). The methoxyl oxygen is derived from molecular oxygen[87] whereas the methyl group originates from methionine.[88] The conversion of (51) into (52) has been demonstrated in extracts of *S. clavuligerus*, and requires the presence of α-ketoglutarate, Fe^{2+}, and a reducing agent, as well as *S*-adenosylmethionine,[89,90] thereby firmly implicating yet another α-ketoglutarate-linked dioxygenase in the secondary metabolism of the β-lactams. This family of

[82] Reviewed by E. P. Abraham, in 'Regulation of Secondary Metabolite Formation', ed. H. Kleinkauf, H. von Dohren, H. Dornauer, and G. Nesemann, VCH, Weinheim, FRG, 1986.
[83] P. B. Loder and E. P. Abraham, *Biochem. J.*, 1971, **123**, 477.
[84] G. S. Jayatilake, J. A. Huddleston, and E. P. Abraham, *Biochem. J.*, 1981, **194**, 645.
[85] S. E. Jensen, D. W. S. Westlake, and S. Wolfe, *Can. J. Microbiol.*, 1983, **29**, 1526.
[86] S. Wolfe, A. L. Demain, S. E. Jensen, and D. W. S. Westlake, *Science*, 1984, **226**, 1386.
[87] J. O'Sullivan, R. T. Alpin, C. M. Stevens, and E. P. Abraham, *Biochem. J.*, 1979, **179**, 47.
[88] J. G. Whitney, D. R. Brannon, J. A. Mabe, and K. J. Wicker, *Antimicrob. Agents Chemother.*, 1972, **1**, 247.
[89] J. O'Sullivan and E. P. Abraham, *Biochem. J.*, 1980, **186**, 613.
[90] J. D. Hood, A. Elson, M. L. Gilpin, and A. G. Brown, *J. Chem. Soc., Chem. Commun.*, 1983, 1187.

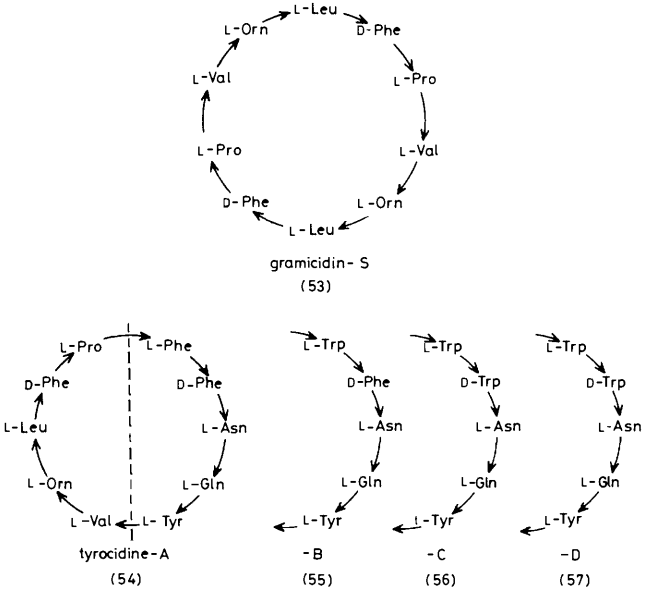


Figure 1 *Structures of gramicidin-S and tyrocidines-A—D*

oxygenases is beginning to emerge as a frequent participant in the oxidation of late stage intermediates in antibiotic biosynthesis, and further examples are described below.

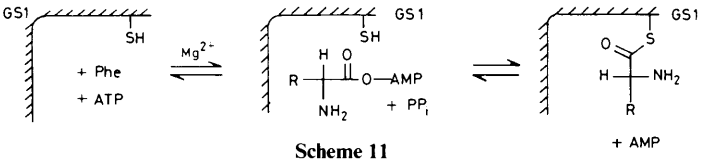
3 Peptide Antibiotics

There are over four hundred[91] different peptide antibiotics known, many produced by microorganisms of the genus *Bacillus*.[92,93] Most of these are cyclic peptides with molecular weights ranging from 270 (bacilysin) to about 4 500 (licheniformin), and many contain amino acids that are not normally found in proteins. Amongst the best studied are gramicidin-S, tyrocidine, linear gramicidin, edeine, bacitracin, colistin, and mycobacillin.[93–96] The biosynthetic machinery is distinct from that involved in protein synthesis (ribosomes and mRNA are not involved), and commonly consists of a multienzyme complex requiring the constituent amino acids, ATP, and Mg^{2+} for activity. Several of these synthetases have been purified by conventional methods, and the prospect that they might

[91] H. Kleinkauf and H. von Dohren, 'Peptide Antibiotics', in 'Comprehensive Biotechnology', Vol. 3, ed. M. Moo-Young, Pergamon Press, Oxford, 1985.
[92] J. Berdy, *Adv. Appl. Microbiol.*, 1974, **18**, 309.
[93] E. Katz and A. L. Demain, *Bacteriol. Rev.*, 1977, **41**, 449.
[94] E. Katz, *Pure Appl. Chem.*, 1971, **28**, 551.
[95] K. Kurahashi, *Ann. Rev. Biochem.*, 1974, **43**, 445.
[96] D. Perlman and M. Bodansky, *Ann. Rev. Biochem.*, 1971, **40**, 449.

Table 7 *Constitution and function of gramicidin-S synthetase and tyrocidine synthetase*

Enzyme	*Fraction*	*Amino acid activated*	*4'-Phospo pantetheine*	*Subunit* M_r
Gramicidin-S synthetase	GSI Light enzyme	L- and D-Phe	0	100 000
	GSII Heavy enzyme	L-Pro, L-Val, L-Orn, L-Leu	1	280 000
Tyrocidine synthetase	I Light enzyme	L- and D-Phe	0	100 000
	II Intermediate enzyme	L-Pro, L- and D-Phe(Trp)	1	3 × 70 000 + 1 × 20 000 = 230 000
	III Heavy enzyme	L-Asn, L-Gln, L-Phe, (Trp, or Tyr), L-Val, L-Orn, L-Leu	1	6 × 70 000 + 1 × 20 000 = 440 000



Scheme 11

be used in bioreactors for cell-free antibiotic production has often been discussed.

The enzymatic formation of these compounds is of great interest, not only with respect to the mechanism of peptide bond formation, but also in order to probe the structural organization of the multienzyme complexes, and the relationship of this to the synthetic ability of the proteins. This is a theme which recurs in other areas of antibiotic biosynthesis, where multienzyme complexes control the synthesis of complex secondary metabolites (*e.g.* macrolides, polyethers, and related polyketides).

A. Gramicidin-S Synthetase and Tyrocidine Synthetase.—Gramicidin-S (53) is a cyclic decapeptide having twofold rotational symmetry due to its head to tail assembly from two identical pentapeptides D-Phe-L-Pro-L-Val-L-Orn-L-Leu, whereas tyrocidines-A,B,C, and D [(54)—(57)] are cyclic decapeptides with the structures shown in Figure 1. The formation of these two cyclic peptides appears to proceed with very similar mechanisms.

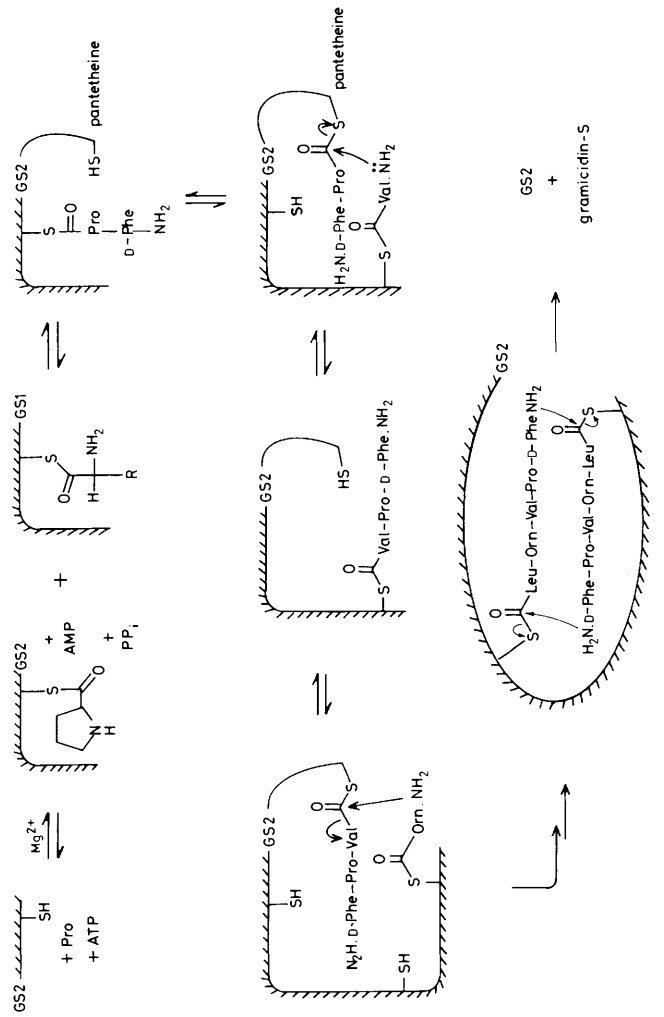
The assembly process is in each case mediated by a multienzyme complex. A recent improvement[97] to the purification procedure for gramicidin-S synthase gives protein with a specific activity of 20—35 nmol/min/mg protein. The gross molecular architecture of the gramicidin-S and tyrocidine multienzyme synthetases is presented in Table 7.

[97] J. Vater, W. Schlumbohm, Z. Palacz, J. Salnikow, A. Gadow, and H. Kleinkauf, *Eur. J. Biochem.*, 1987, **163**, 297.

For gramicidin production, two multifunctional polypeptide chains are necessary. The smaller of these, GS1 (M_r 100 000), can accept and activate either L-Phe or D-Phe in an ATP-dependent process, which proceeds *via* an enzyme-bound aminoacyladenylate intermediate (Scheme 11), leaving the amino acid directly linked to the protein as a thioester.[98,99] In support of this notion, the phenylalanine can be released from the enzyme by alkaline hydrolysis,[100] affording a 70:30 mixture of the *R* and *S* isomers. The second heavier protein GS2 (M_r 280 000) accepts the activated Phe from GS1, only as the *R*-isomer, and then completes the assembly of the antibiotic. This protein also contains one mole of 4′-phosphopantetheine whose thiol group is believed to play a key role in the synthesis. A model to explain this assembly was first proposed by Lipmann and coworkers,[98] in which the synthesis proceeds without free intermediates from the N-terminus following the path shown in Scheme 12. The amino acids L-Pro, L-Val, L-Orn, and L-Leu are added to the growing chain, singly, in a fixed order, such that omission of one amino acid leads to cessation of the elongation process. The first peptide bond is formed by the transfer of thioester bound D-Phe on GS1 to the free amine-N of L-Pro, itself held as a thiol ester on GS2. The resulting dipeptide is then transferred to the thiol group of the pantetheine side arm. This side arm may now act to transfer the growing chain to the next active site, where the next thiol ester bound amino acid, L-Val, is waiting. Its free amino group accepts the dipeptide unit, and the resulting tripeptide is transferred again to the pantetheine swinging arm, thereby allowing transport to the next active site, and so on. This assembly continues until a tetrapeptide unit is transferred to the free amine of thiol ester bound L-Leu, whereupon the pentapeptide participates in a rapid cyclization resulting in release of gramicidin-S. Exactly how the timing of the second pentapeptide is organized remains unclear; it is possible that the first pentapeptide is transferred to a holding site on the heavy enzyme, until assembly of the second one is complete. The four amino acid activating sites are arranged on the single polypeptide of M_r 280 000, in domains of approximate M_r 70 000, which can be progressively released upon limited proteolysis.[91]

A different architecture is seen with tyrocidine synthetase,[101] where now in addition to a so-called 'light enzyme' (LE) of M_r 100 000 which accepts and activates L- or D-Phe, there are two other protein complexes involved (Table 7). One of these, the intermediate enzyme (IE, M_r 230 000) activates and incorporates L-Pro and L- or D-Phe, whereas the other, the heavy enzyme (HE, M_r 460 000) activates and incorporates L-Asn, L-Gln, L-Phe, L-Val, L-Orn, and L-Leu. The subunit composition of the tyrocidine synthetase has been explored,[101–103] and here a significant difference is seen from gramicidin synthetase. Both the IE and

[98] F. Lipmann, *Acc. Chem. Res.*, 1973, **6**, 361.
[99] J. Vater, N. Mallow, S. Gerhardt, A. Gadow, and H. Kleinkauf, *Biochemistry*, 1985, **24**, 2022.
[100] R. Roskoski, H. Kleinkauf, W. Gevers, and F. Lipmann, *Biochemistry*, 1970, **9**, 4846.
[101] S. G. Lee, R. Roskowski, K. Bauer, and F. Lipmann, *Biochemistry*, 1973, **12**, 398.
[102] R. Roskowski, W. Gevers, H. Kleinkauf, and F. Lipmann, *Biochemistry*, 1970, **9**, 4839.
[103] S. G. Lee and F. Lipmann, *Proc. Nat. Acad. Sci. USA*, 1977, **74**, 2343.


GS2
SH
+ Pro
+ ATP
Mg2+
GS2
+ AMP
+ PP i
GS1
GS2
pantetheine
GS2
SH
N2H.D-Phe-Pro-Val
Orn.NH2
GS2
SH
H2N.D-Phe-Pro-Val
GS2
HS
GS2
pantetheine
GS2
SH
H2N.D-Phe-Pro
Val.NH2
GS2
Leu-Orn-Val-Pro-D-Phe NH2
H2N.D-Phe-Pro-Val-Orn-Leu
GS2
+
gramicidin-S

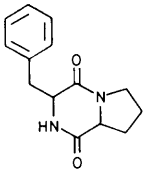
Scheme 12

the HE complexes can be dissociated to afford proteins of M_r 90 000 and 70 000. The IE complex liberates these in a 1:2 ratio and the HE in a 1:5 ratio. The M_r 90 000 subunit can be further dissociated into an additional M_r 70 000 subunit, and a protein of M_r 20 000, which contains a covalently bound 4′-phosphopantetheine moiety (analogous to the acyl carrier protein of fatty acid synthetase). Thus, the small pantetheine$^+$ protein is shared by three amino acid activating subunits each of M_r 70 000 in IE, and by six in HE. It seems to be no coincidence that the number of 70 000 dalton subunits exactly matches the number of amino acids accepted and coupled by the IE and HE proteins. When the 90 000 dalton complex is incubated with the light enzyme in the presence of Phe and Pro, a D-Phe-Pro dipeptide was formed[103] that cyclized to afford the D-Phe-Pro diketopiperizine (58). On the other hand, the 70 000 dalton subunit alone could not generate this by-product under identical conditions. This seems to confirm the view that activated amino acids are translocated from a protein thiol on the M_r 70 000 subunits, to the pantetheine thiol, during chain elongation, and is consistent with broadly the same model of chain assembly as discussed earlier for gramicidin-S synthetase (see Scheme 12). This would involve, firstly, activation of Phe on LE and transfer of D-Phe to the free amine of L-Pro bound to IE. The dipeptide is transferred to the acyl carrier protein and sequentially three more amino acids are added whilst bound to IE. The tetrapeptide is then transferred to the HE, where the remaining six amino acids are sequentially added to complete the peptide chain. Cyclization of the decapeptide is apparently relatively slow, since the free linear decapeptide can be recovered from the complex after treatment with alkali. As with the gramicidin synthetase, omission of the constituent amino acids in an *in vitro* assay leads to cessation of chain synthesis, and leaves peptides of intermediate length, that also can be recovered from the protein by mild hydrolysis.

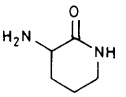
Side reactions are a common feature of these enzymic processes. Thus the diketopiperazine (58) is formed by the gramicidin-S synthetase when it is supplied with only the first two substrate amino acids.[104] In a similar manner, L-ornithine is rapidly converted[105] into the piperidone (59) at its binding site on GS2, and the D-Phe-Pro-Val-cyclo-Orn (60) was obtained[97] as a product of the multienzyme system by omission of L-Leu from the assay cocktail. The substrate specificities of both the gramicidin-S and tyrocidine synthetases have been examined in detail, particularly with a view to producing novel antibiotics, and these data are reviewed elsewhere.[104] It is found that both enzymes have a broad specificity, and the structural variants that are generated most successfully involve the substitution of one aromatic amino acid for another, or by structural variations in the branched chain amino acid residues.

At the present time little is known of the primary sequences of these two multienzyme complexes, although progress is being made towards cloning their structural genes.[118] Once achieved this will allow the subcloning of individual

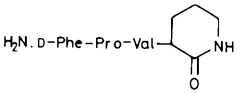
[104] H. Kleinkauf and H. von Dohren, in 'Trends in Antibiotic Research', Japan Antibiotics Research Association, 1982, Tokyo.



(58)



(59)

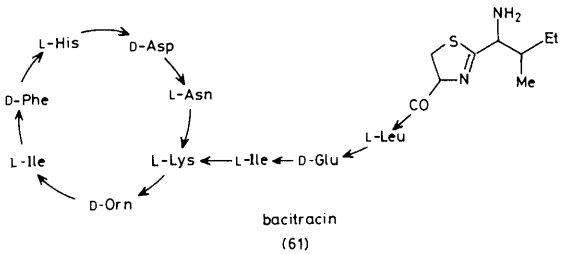


(60)

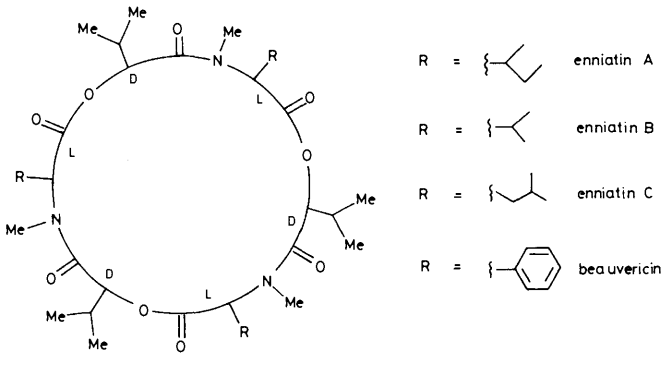
amino acid activating units, their over expression, and possibly their union in novel combinations to produce new derivatives of these peptide antibiotics.[106,107]

B. Bacitracin Synthetase.—Although several bacitracins have been discovered, the most completely investigated is bacitracin-A (61), a dodecapeptide containing four D-amino acid residues (Glu, Orn, Phe, and Asn), a cyclic hexapeptide, and a thiazoline ring. This antibiotic is synthesized from its constituent amino acids, by a bacitracin synthetase of approximately 800 000 daltons. The complex has been resolved into three components,[108,109] named in the order they elute from a sepharose affinity column,[110,111] and each contains a covalently bound 4′-phosphopantetheine moiety. Component I is a multifunctional protein composed of a single polypeptide chain[112] with M_r 140 000, which activates L-Ileu, L-Cys, L-Leu, and L-Glu in the presence of ATP and Mg^{2+}, suggesting the involvement of this subunit in the synthesis of the linear N-terminal portion of bacitracin, including the construction of the thiazoline ring between Cys and Leu. A second fraction, component II, has a similar molecular weight, but only activates L-Lys and L-Orn, whereas the third, component III, has a molecular weight of about 380 000 daltons, and activates L-Phe, L-His, L-Asp, L-Asn, and L-Ileu (or Val).[111] Again, amino acid activation requires ATP and Mg^{2+}, and proceeds *via* an aminoacyladenylate, to afford amino acids linked to the protein as thioesters. As yet very little more is currently known concerning how the nominal 53 catalytic functions needed for bacitracin formation are orchestrated on this complex.

[105] A. Gadow, J. Vater, W. Schlumbohn, Z. Palacz, J. Salinkow, and H. Kleinkauf, *Eur. J. Biochem.*, 1983, **132**, 229.
[106] M. Krause, M. A. Marahiel, H. von Dohren, and H. Kleinkauf, *J. Bacteriol.*, 1985, **162**, 1120.
[107] H. Kleinkauf and H. von Dohren, in 'Regulation of Secondary Metabolite Formation', ed., H. Kleinkauf, H. von Dohren, H. Dornauer, and G. Neseman, Proceedings of the 16th Hoechst Workshop Conference, 1985, VCH, Weinheim, FRG, 1986.
[108] O. Froshov, *FEBS Lett.*, 1974, **44**, 75.
[109] I. Roland, O. Froshov, and S. G. Laland, *FEBS Lett.*, 1977, **84**, 22.
[110] I. Roland, O. Froshov, and S. G. Laland, *FEBS Lett.*, 1975, **60**, 305.
[111] H. Ishihara, Y. Endo, S. Abe, and K. Shimura, *FEBS Lett.*, 1975, **50**, 43.
[112] I. Ogawa, H. Ishihara, and K. Shimura, *FEBS Lett.*, 1981, **124**, 197.



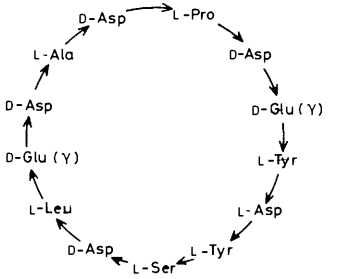
bacitracin
(61)



(62)

C. Enniatin Synthetase.—The enniatins are a group of structurally homologous cyclic depsipeptides,[113] produced by several strains of the eukaryote *Fusarium*. They consist of three residues of the branched chain α-amino acids L-Val, L-Leu, or L-Ileu, and three residues of D-2-hydroxyisovaleric acid. These constituents are linked through ester and amide bonds in an alternating fashion, and several of the amide nitrogens are *N*-methylated, as in (62). The multienzyme system catalysing enniatin formation has been purified to homogeneity,[114] and consists of a single polypeptide chain of M_r 250 000. The enzyme contains 4′-phosphopantetheine as a covalently bound cofactor, and requires *S*-adenosylmethionine for full activity. Omission of SAM from assays results in the formation of unmethylated enniatins at 10% the reaction rate. The role of the 4′-phosphopantetheine is assumed to be once again as a swinging arm to transfer the growing chain between active sites. Indeed, evidence that there are clear structural similarities between gramicidin synthetase GS2 and enniatin synthetase follows from the observation that antibodies raised against the former cross-react with the latter in a Western blot analysis.[107] Also, sheep antibodies against enniatin synthetase cross-react with gramicidin synthetase.[107] Both antibodies are able to inhibit amino acid or hydroxy acid activation reactions catalysed by

[113] V. S. Malik, *Adv. Appl. Microbiol.*, 1982, **28**, 27.
[114] R. Zocher, U. Keller, and H. Kleinkauf, *Biochemistry*, 1982, **21**, 43.



mycobacillin

(63)

these enzymes. A more detailed structural knowledge of the two proteins is now required, which should lead to a clearer understanding of these structural relationships, and their function in synthesis.

D. Mycobacillin Synthetase.—Mycobacillin (63) is a cyclic peptide antibiotic containing 13 amino acid residues, and is produced by *Bacillus subtilis B3*. Most of the peptide bonds are of the normal type, except the D-Glu residues which are coupled through the γ-carboxyl rather than the α-carboxyl. The purification of the components of the mycobacillin synthetase complex has been reported recently.[115] These were isolated in three fractions, A, B, and C after gel filtration, with molecular weights of approximately 252 000, 190 000, and 105 000, respectively. The synthesis appears to occur[116] by a three-step process, the enzyme fraction A synthesizing a pentapeptide in the first step, fraction B a nonapeptide in the second step, and fraction C completing the assembly in the third step.[117] Interestingly, none of the fractions seems to contain a 4′-phosphopantetheine side arm, although the activation of each constituent amino acid again requires ATP.

E. Actinomycin.—The actinomycins are produced by several strains of the Gram positive bacteria *Streptomyces* (Scheme 13). The broad features of their biosynthesis have been established, and several enzymes on the pathway have been purified to homogeneity. Thus actinomycin-C is formed by the oxidative coupling of two 4-methyl-3-hydroxyanthranilic acid (4-MHA) pentapeptide lactones (64), as catalysed by a phenoxazinone synthase, isolated,[119] cloned[120]

[115] S. K. Gosh, N. K. Mukhopadhyay, S. Majumder, and S. K. Bose, *Biochem. J.*, 1986, **235**, 81.
[116] S. K. Gosh, S. Majumder, N. K. Mukhopadhyay, and S. K. Bose, *Biochem. J.*, 1986, **240**, 265.
[117] S. K. Gosh, S. Majumder, N. K. Mukhopadhyay, and S. K. Bose, *Biochem. J.*, 1985, **230**, 785.
[118] M. A. Marahiel, M. Krause, and H. J. Skarpeid, *Mol. Gen. Genet.*, 1985, **201**, 231; M. Krause and M. A. Marahiel, *J. Bacteriol.*, 1988, **170**, 4669.
[119] H. A. Choy and G. H. Jones, *Arch. Biochim. Biophys.*, 1981, **211**, 55.
[120] G. H. Jones and D. A. Hopwood, *J. Biol. Chem.*, 1984, **259**, 14151 & 14158.

(*vide infra*) from *S. antibioticus*, and overproduced in *S. lividans*. The enzyme has a subunit molecular weight of 88 000, with the active forms existing as a mixture of dimers and hexamers,[119] with approximate molecular weights of 180 000 and 530 000 kDa. The enzyme is a copper-containing protein and catalyses the oxidation of a wide variety of aminophenols to phenoxazinones.[121] The mechanism of the dimerization has been probed using substituted aminophenols, which block the synthesis of phenoxazinone at various stages (Scheme 14). Based on these results[121] a mechanism has been proposed, which involves a sequence of three consecutive aminophenol oxidations, as shown in Scheme 14.

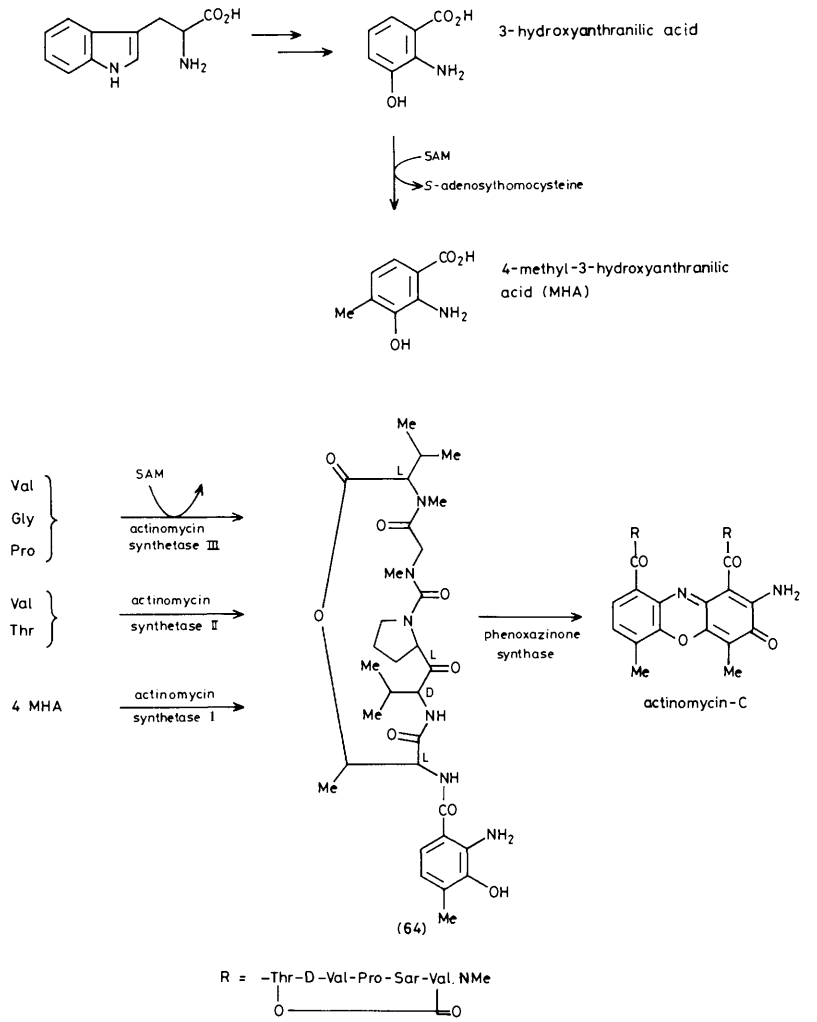
The 4-MHA-pentapeptide lactone (64) is assembled in stages from 4-MHA and the requisite amino acid derivatives. Two multifunctional enzymes called actinomycin synthetases II and III are responsible for the assembly of the five amino acids in the peptide lactone chains.[122] Actinomycin synthetase II activates threonine and valine, found in positions 1 and 2, as thioesters, whereas actinomycin synthetase III activates in a similar way the remaining three amino acids, proline, glycine, and valine. In addition, the latter enzyme has the methyltransferase functions involved in the *N*-methylation of enzyme-bound glycine and valine, and catalyses the formation of the lactone ring at the expense of ATP. The two enzymes each consist of single polypeptide chains of M_r 225 000 and 280 000, respectively. In addition, actinomycin synthetase I is a 4-MHA activating enzyme, composed of a single chain of relative M_r 47 000, which is involved in the attachment of the aromatic carboxylic acid to the pentapeptide chain.[123]

The 4-MHA itself appears to be a product of tryptophan metabolism. A 3-hydroxyanthranilate 4-methyltransferase has been purified to near homogeneity[124] (Scheme 13) from *S. antibioticus*. This enzyme converts 3-hydroxyanthranilate (3-HAA) into 4-MHA, with *S*-adenosylmethionine providing the methyl group. This interesting *C*-methyltransferase is a single polypeptide of about 36 000 kDa. In turn, the 3-HAA may be derived from tryptophan, *via N*-formylkynurenine, kynurenine, and 3-hydroxykynurenine. The first enzyme in this sequence, tryptophan di-oxygenase, has also been purified[125] to near homogeneity from an actinomycin-D-producing strain of *S. parvulus*, and shown to have a native M_r of 88 000. The enzyme is inhibited by CN^-, and seems to contain a tightly bound heme prosthetic group. At least in this respect, it appears to be similar to tryptophan 2,3-dioxygenases previously isolated from other microbial, and mammalian sources.

4 Macrolide, Polyether, and Related Polyketide Antibiotics

The polyketide family of secondary metabolites is taken here to include all of the

[121] C. E. Barry, P. G. Nayar, and T. P. Begley, *J. Am. Chem. Soc.*, 1988, **110**, 3333.
[122] U. Keller, *J. Biol. Chem.*, 1987, **262**, 5852.
[123] U. Keller, H. Kleinkauf, and R. Zocher, *Biochemistry*, 1984, **23**, 1479.
[124] F. Fawaz and G. H. Jones, *J. Biol. Chem.*, 1988, **263**, 4602.
[125] M. J. M. Hitchcock and E. Katz, *Arch. Biochim. Biophys.*, 1988, **261**, 148.

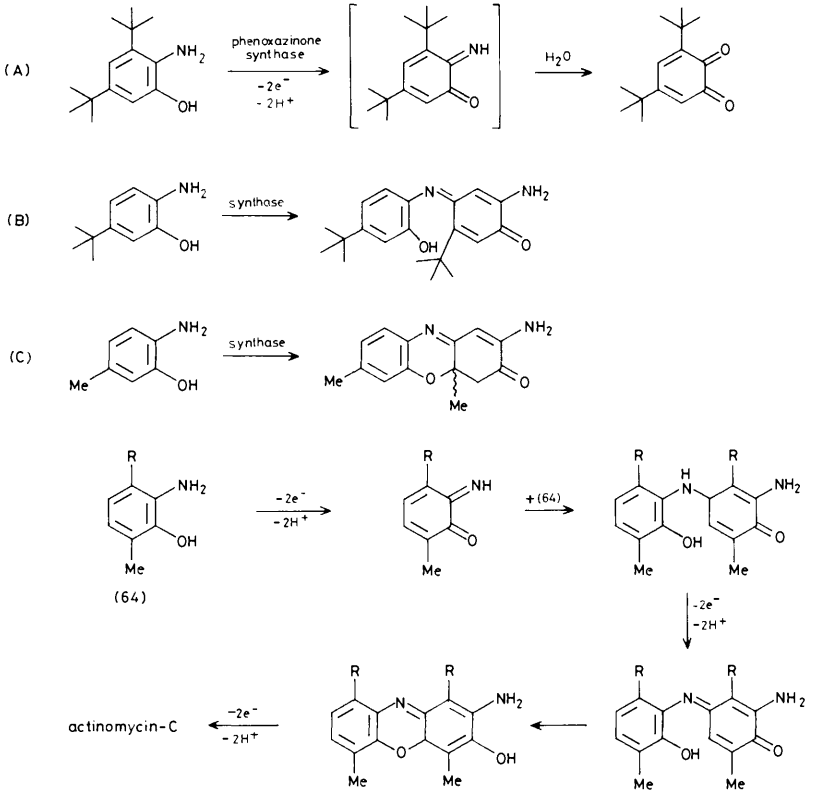


Scheme 13

structures assembled by the head-to-tail coupling of acetate, propionate, and/or butyrate units, as elaborated in the classical studies of Birch and co-workers[126] looking at aromatic polyketides. Therefore, this is most probably the largest single family of natural products, and certainly the one whose biosynthesis is currently least well defined from an enzymological viewpoint.

When considering the macrolide and polyether antibiotics, an important

[126] A. J. Birch and F. W. Donovan, *Aust. J. Chem.*, 1953, **6**, 360.



Scheme 14

feature of their biosynthetic pathways appears to be a requirement for a carbon chain building process capable of generating a long, branched and oxygenated fatty acid backbone. Once this chain assembly is complete the backbone may be modified in a variety of ways, including by the addition of sugar residues, and other oxidative processes utilizing molecular oxygen. Even within the classical structural groupings, the large number of known polyethers (>80) and macrolides (>150) is strongly indicative of a biosynthetic theme upon which Nature has played numerous variations to generate the diversity of observable regio- and stereo-chemistries. On the other hand, when structural comparisons are made within each family an intriguing pattern of structural and stereochemical homologies can be perceived. This is illustrated most effectively in the Celmer model of macrolide structure,[127] and the recent Cane–Celmer–Westley model of polyether structure and stereochemistry.[128] Although these structural similarities must arise, to some extent, from a biological pressure that requires the product to

[127] W. P. Celmer, *Pure Appl. Chem.*, 1971, **28**, 413.

Table 8 *Simplified view of the constitution and functional units in the fatty acid synthase complex*

Organism	*Subunit type and Mol. Wt.*		*Reference*
Most bacteria and plant chloroplasts	6—7 separate enzymes + acyl carrier protein		129,130
Yeast	2 dissimilar subunits		130,131
	α 212 000	condensing enzyme acyl carrier site β-ketoacyl reductase	
	β 203 000	enoyl reductase acetyl transferase dehydratase malonyl and palmitoyl transferases	
	active form $\alpha_6\beta_6$	M_r 2.4 × 10^6	
Birds, mammals	Single polypeptide, fully funcational as a dimer α_2 M_r 4—5 × 10^6 contains an acyl carrier site		130—135

possess antibiotic activity, the underlying enzymic and genetic bases for them are at present unclear.

The fundamental chemistry relating the biosynthesis of these metabolites is most likely analogous, in many respects, to that catalysed by the fatty acid synthase (FAS) complex (Figure 2). Important progress has been made over the past five years in relating the structure of prokaryotic and eukaryotic fatty acid synthases to the reactions catalysed by these multienzyme systems. This has revealed a remarkable variety of structural forms (Table 8) for the complexes in different species, in spite of a basic similarity in the pathway of fatty acid synthesis.

The animal FAS (a type I FAS) exists as a dimer of identical subunits,[132–135] each with M_r 267 000, and each containing seven catalytic centres and an acyl carrier site. Proteolytic cleavage patterns indicate that each subunit is arranged into three major globular domains interconnected by polypeptide bridges that are susceptible to protease cleavage (Figure 3). Domain I contains the NH_2-terminal end of the polypeptide and the catalytic sites of β-ketoacyl synthase (condensing enzyme) as well as the acetyl- and malonyl-transacylases. Domain II

[128] D. E. Cane, W. D. Celmer, and J. W. Westley, *J. Am. Chem. Soc.*, 1983, **105**, 3594.
[129] A. J. Fulco, *Prog. Lipid Res.*, 1983, **22**, 133.
[130] A. D. McCarthy and D. G. Hardie, *Trends in Biochem. Res.*, 1984, 60.
[131] S. J. Wakil, J. K. Stoops, and V. C. Joshi, *Ann. Rev. Biochem.*, 1983, **52**, 537.
[132] J. S. Mattick, Y. Tsukamoto, J. Nickless, and S. J. Wakil, *J. Biol. Chem.*, 1983, **258**, 15291.
[133] J. S. Mattick, J. Nickless, M. Mizugaki, C.-Y. Yang, S. Uchiyama, and S. J. Wakil, *J. Biol. Chem.*, 1983, **258**, 15300.
[134] H. Wong, J. S. Mattick, and S. J. Wakil, *J. Biol. Chem.*, 1983, **258**, 15305.
[135] Y. Tsukamoto, H. Wong, J. S. Mattick, and S. J. Wakil, *J. Biol. Chem.*, 1983, **258**, 15312.

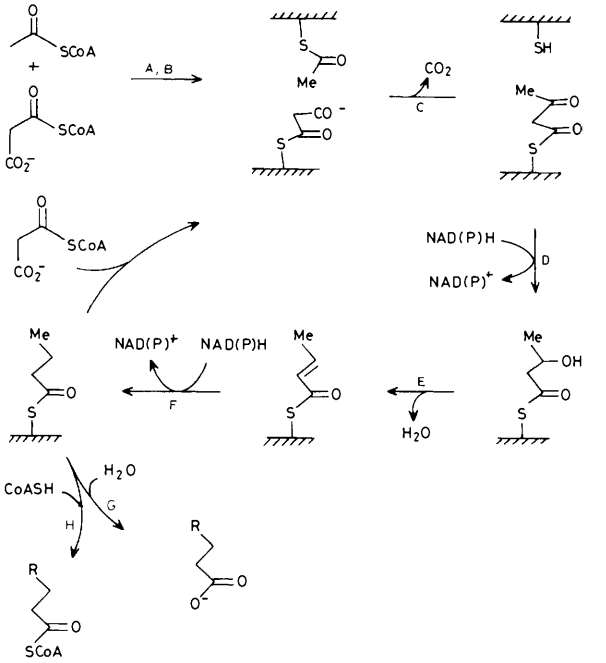


Figure 2 *Activities of the fatty acid synthase complex. A, B = acetyl, malonyl transferases; C = β-ketoacyl synthase (condensing enzyme); D = β-ketoacyl reductase; E = dehydratase; F = enoyl reductase; G = thioesterase, or H = palmitoyl transferase*

contains the β-ketoacyl and enoyl reductases, the dehydratase, and the 4′-phosphopantetheine prosthetic group of the acyl carrier protein site. Domain III includes the carboxyl terminus and contains the thioesterase activity that releases the fatty acyl group when the chain reaches C_{16} in length. Dissociation of the enzyme into its component monomers results in retention of all the individual activities except that of the condensing enzyme. Other evidence[131] suggests that this activity requires the juxtapositioning of two thiols, one on the condensing enzyme, and the other the cysteamine-SH of the 4′-phosphopantetheine in the acyl-carrier site of the *other subunit* (see Figure 3). In contrast to this, most bacteria contain a type II FAS in which each reaction is catalysed by a discrete, separable polypeptide, and the acyl carrier site is now located on a small protein of M_r around 10 000; the acyl carrier protein. In *E.coli* these polypeptides are the products of only distantly related genes, whereas in mammals the FAS dimer is derived from a single large mRNA. The yeast, *Saccharomyces cerevisia* represents an intermediate case in which the FAS consists of two classes of polypeptide, components α and β, carrying three and five of the catalytic functions, respectively. Although the αβ complex contains all the activities required for

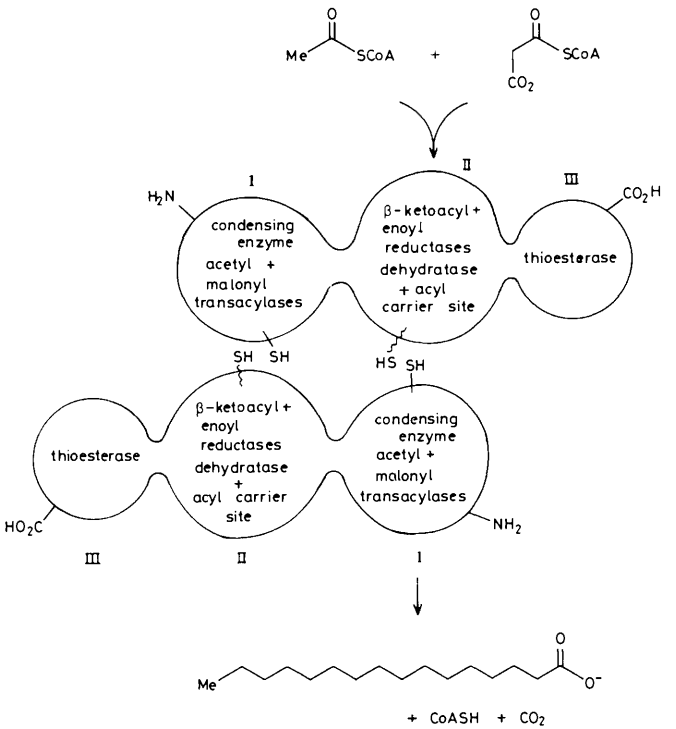


Figure 3 *A model of the type-I fatty acid synthase complex*

palmitoyl-CoA synthesis, the active form is a hexamer, $\alpha_6\beta_6$, with a molecular weight of 2.4×10^6.

Whereas the carbon chain of fatty acids is assembled by repetition of a cycle comprising condensation–reduction–dehydration–reduction steps, the processes occurring during polyketide biosynthesis are operationally more complex. Thus FAS normally accepts acetyl-CoA as a starter unit, while many polyketide synthases (PKSs) accept other starter units, and some show a lack of specificity, and may accept one of several. Further, the fatty acid chain is extended always with malonyl-CoA, whereas the PKS can select malonyl-CoA, methylmalonyl-CoA, or ethylmalonyl-CoA (and sometimes other units) at each stage of the assembly, according to a predetermined order. Moreover, methylmalonyl-CoA and ethylmalonyl-CoA exist as pairs of diastereomers that may also be distinguished by the PKS. Finally, the FAS constructs a fully saturated chain, whereas a seemingly endless variety of functional groups (ketones, alcohols, double bonds as well as saturated linkages) and stereochemical configurations may be found in the backbone of any given PKS product [illustrated in Figure 4 for the case of the putative polyketide precursor (65) in monensin-A biosyn-

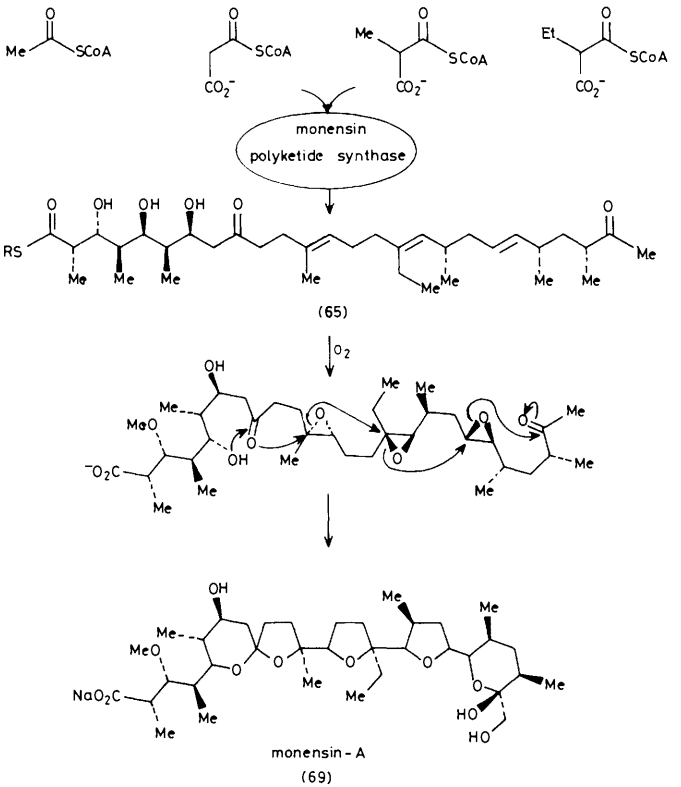
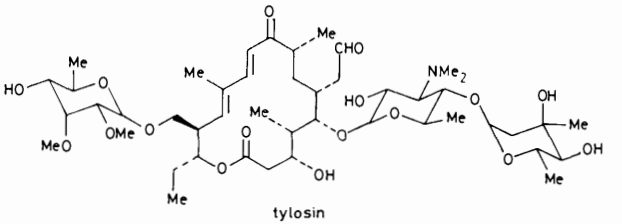


Figure 4 *The functional complexity of a typical polyether polyketide synthase complex* (cf. *Figure* 3)

thesis[136]]. Moreover each assembly proceeds with a remarkable degree of fidelity, with only rare lapses in specificity. It is these additional complexities of PKS function that represent the fundamental challenge to be addressed in studies of the structure of these multicomponent proteins. How is the information that controls these sets of choices encoded and interpreted in such a way that each PKS typically catalyses the synthesis of a single type of carbon chain, with a specific pattern of functional group substitutions?

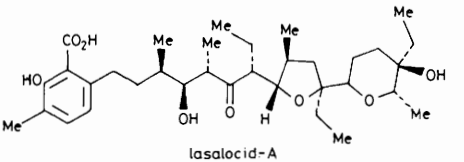
A. Macrolide Antibiotics.—This discussion is relevant not only to the classical groups of 12-, 14-, and 16-membered macrolide antibiotics, but also to other polyketides containing a macrocyclic lactone, including the avermectins, milbemycins, ansamycins, polyene macrolides, boromycin, aplasmomycin, and

[136] D. E. Cane, T. C. Liang, and H. Hasler, *J. Am. Chem. Soc.*, 1982, **104**, 7274.



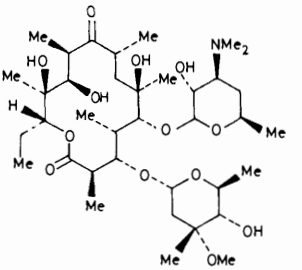
tylosin

(66)



lasalocid-A

(67)



erythromycin-A

(68)

chlorothricin amongst others. An excellent review of the enzymic aspects of macrolide biosynthesis has appeared recently.[137]

The biosynthesis of a typical macrolide, such as tylosin (66), proceeds in readily discernible phases, which include in this case: (*a*) assembly of the macrolide carbon backbone; (*b*) preparation of relevant monosaccharides; (*c*) oxidation of the macrolide backbone, utilizing molecular oxygen; (*d*) attachment of sugar residues to the macrolide; (*e*) final modifications, *e.g.* methylation on oxygen or nitrogen.

(i) *Assembly of the Carbon Backbone.* To date there have been no reports of successful attempts to detect and isolate the polyketide synthases active in macrolide and polyether antibiotic biosynthesis. Evidence that their mode of action parallels that of the well studied FASs has come indirectly from a number of sources. In several systems the feeding of labelled precursors to

[137] J. Neuzil and Z. Hostalek, *Folia Microbiol.*, 1986, **31**, 402.

whole-cell cultures has revealed some details of the carbon–carbon bond forming steps, catalysed by a condensing enzyme in the PKS complex. These include the incorporation of [2-$^2H_2$, 2-^{13}C]propionate into lasalocid-A[138,139] (67) and erythromycin-A[140] (68), and the incorporations of [2-$^2H_2$]-, (*R*)-[2-$^2H_1$]- and (*S*)-[2-$^2H_1$]-propionates into monensin-A[141,142] (69). Where retention of deuterium label in the antibiotic could be detected, its location is always at chiral methine sites whose absolute configurations are consistent with the processing of the propionate firstly to propional-CoA, and then by carboxylation to (*S*)-methylmalonyl-CoA, with the incorporation of the latter into the backbone occurring by a decarboxylative-condensation proceeding with inversion of configuration (see Scheme 15). This is precisely the stereochemical outcome of the analogous step in fatty acid biosynthesis, catalysed by the condensing enzyme.[143–146] Unfortunately, it was not possible to determine whether (*R*)-methylmalonyl-CoA is also utilized in a similar way by the PKSs, during chain elongation (Scheme 16) [because of the difficulties in generating the relevant labelled (*R*)-methylmalonyl-CoA *in vivo*], although this would provide an elegant mechanism for determining the sense of chirality at these centres as they are introduced into the backbone.[147]

The frequent use of methylmalonyl-CoA by these PKS enzymes underscores the importance of this primary metabolite as a potentially limiting precursor for secondary metabolite production. Three important routes to this building block have been characterized in Streptomycetes (Scheme 17); propionyl-CoA carboxylase[148] and methylmalonyl-CoA mutase[149] have been purified from *S. erythreus* and are implicated in erythromycin production, whereas n-butyrate and isobutyrate are known to be efficient precursors of methylmalonyl-CoA in several Streptomycetes.[150–152] The isobutyryl-CoA should be converted into methylmalonyl-CoA *in vivo* by oxidation of its *pro-S* methyl group.[153] A novel coenzyme-B_{12}-dependent mutase has also been partially purified[154] from *S.*

[138] C. R. Hutchinson, M. M. Sherman, A. G. McInnes, J. A. Walter, and J. C. Vederas, *J. Am. Chem. Soc.*, 1981, **103**, 5956.
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[140] D. E. Caine, T. C. Liang, P. B. Taylor, C. Chang, and C. C. Yang, *J. Am. Chem. Soc.*, 1986, **108**, 4957.
[141] G. R. Sood, J. A. Robinson, and A. A. Ajaz, *J. Chem. Soc., Chem. Commun.*, 1984, 1421.
[142] G. R. Sood, D. M. Ashworth, A. A. Ajaz, and J. A. Robinson, *J. Chem. Soc., Perkin Trans. 1*, 1988, 3183.
[143] B. Sedgwick and J. W. Cornforth, *Eur. J. Biochem.*, 1977, **75**, 465.
[144] B. Sedgwick, J. W. Cornforth, S. J. French, R. T. Gray, E. Kelstrup, and P. Willadsen, *Eur. J. Biochem.*, 1977, **75**, 481.
[145] B. Sedgwick, C. Morris, and S. J. French, *J. Chem. Soc., Chem. Commun.*, 1978, 193.
[146] V. E. Anderson and G. G. Hammes, *Biochemistry*, 1984, **23**, 2088.
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[148] A. R. Hunaiti and P. E. Kolattukudy, *Arch. Biochim. Biophys.*, 1982, **216**, 362.
[149] A. R. Hunaiti and P. E. Kolattukudy, *Antimicr. Agents Chemother.*, 1984, **25**, 173.
[150] S. Omura, K. Tsuzuki, Y. Tanaka, H. Sakikibara, M. Aizawa, and G. Lukacs, *J. Antibiot.*, 1983, **36**, 614.
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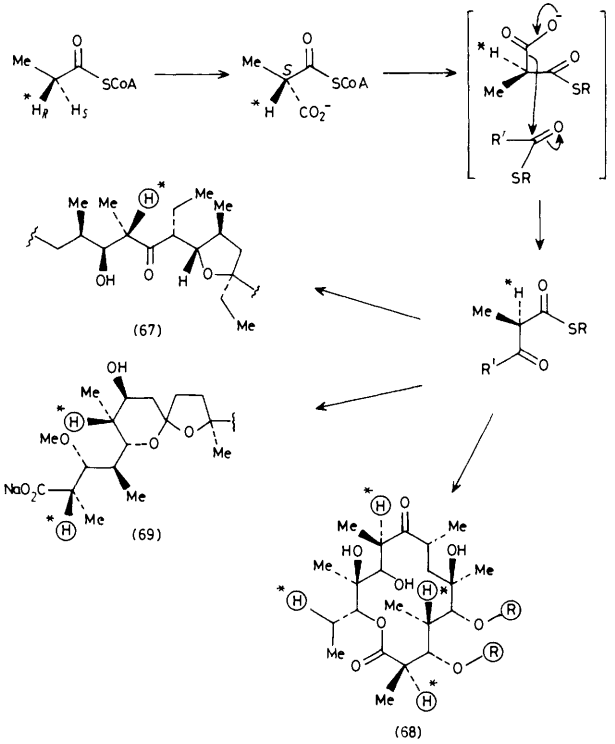
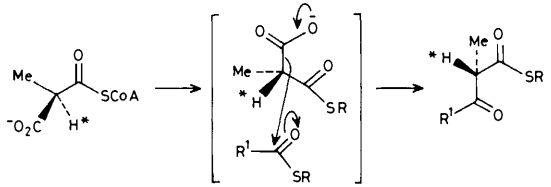
[152] M. M. Sherman, S. Yue, and C. R. Hutchinson, *J. Antibiot.*, 1986, **39**, 1135.
[153] D. Gani, D. O'Hagan, K. Reynolds, and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1985, 1002.
[154] G. Brendelberger, J. Retey, D. M. Ashworth, K. Reynolds, F. Willenbrock, and J. A. Robinson, *Angew. Chem., Int. Ed. Engl.*, 1988, **27**, 1089.



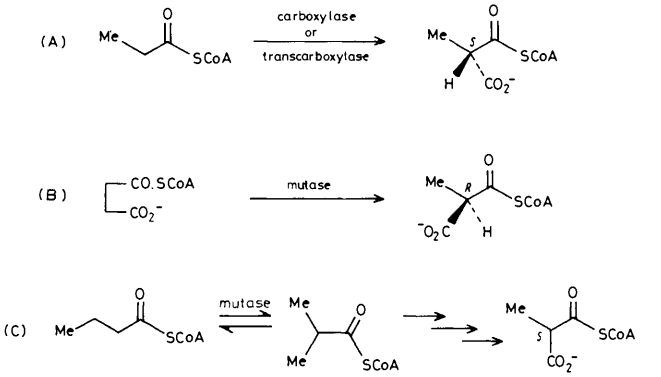
Scheme 15



Scheme 16

cinnamonensis that catalyses the reversible rearrangement[155,156] of isobutyryl-CoA to n-butyryl-CoA, thereby forming an important metabolic link between straight chain and branched chain fatty acid metabolism in these organisms.[157] A malonyl-CoA decarboxylase has been purified[158] from *S. erythreus*, and it has been

[155] K. Reynolds and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1985, 1831.
[156] K. Reynolds, D. Gani, and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1986, 1334.
[157] K. Reynolds, D. O'Hagan, D. Gani, and J. A. Robinson, *J. Chem. Soc., Perkin Trans. 1*, 1988, 3195.
[158] A. R. Hunaiti and P. E. Kolattukudy, *Arch. Biochim. Biophys.*, 1984, **229**, 426.



Scheme 17

suggested that this may also play a role in antibiotic production. It seems well established that antibiotic production in Streptomycetes takes place predominantly after the initial period of rapid cell growth, when the activity of the Krebs cycle and fatty acid biosynthesis become lower. It is during this period that the major pathways of catabolism (of amino acids, and fatty acids) also become most active, and it should be these pathways, in addition to the Krebs cycle, that provide the bulk of the carbon source for secondary metabolite formation.

Following each carbon–carbon bond forming step in chain assembly, the PKS must decide whether to continue the synthesis by adding another building block to the chain, leaving behind a ketone—a process which when used iteratively would generate a poly-β-ketide—or to alter the ketone first, by one or more of a series of reduction–dehydration–reduction steps. Some evidence that it is the latter, so-called processive strategy,[159] that is employed has again come from whole-cell feeding experiments. Tylosin is an important 16-membered ring macrolide whose biosynthesis has been well studied (see refs. 172 and 179). The first formed macrolide is tylactone (70), which is assembled from propionyl-CoA (starter unit), two malonyl-CoAs, four methylmalonyl-CoAs, and an ethylmalonyl-CoA (Scheme 18). No intermediates in the chain assembly have been detected, and all mutants of the producing strain that are blocked in the stages leading up to tylactone fail to participate in pairwise co-synthesis experiments, again indicating that no intermediates in the chain assembly are ever released free from a carrier protein. The macrolide tylactone (70) should therefore be the product of this macrolide PKS, and may be assembled by either of the routes outlined in Scheme 18. In order to distinguish between these, the putative intermediates (71), (72), and (73) were synthesized in labelled form, activated as acetylcysteamine thioesters, and were shown to be incorporated into tylactone, when administered to shake flask cultures of the producing

[159] S. Yue, J. S. Duncan, Y. Yamamoto, and C. R. Hutchinson, *J. Am. Chem. Soc.*, 1987, **109**, 1253.

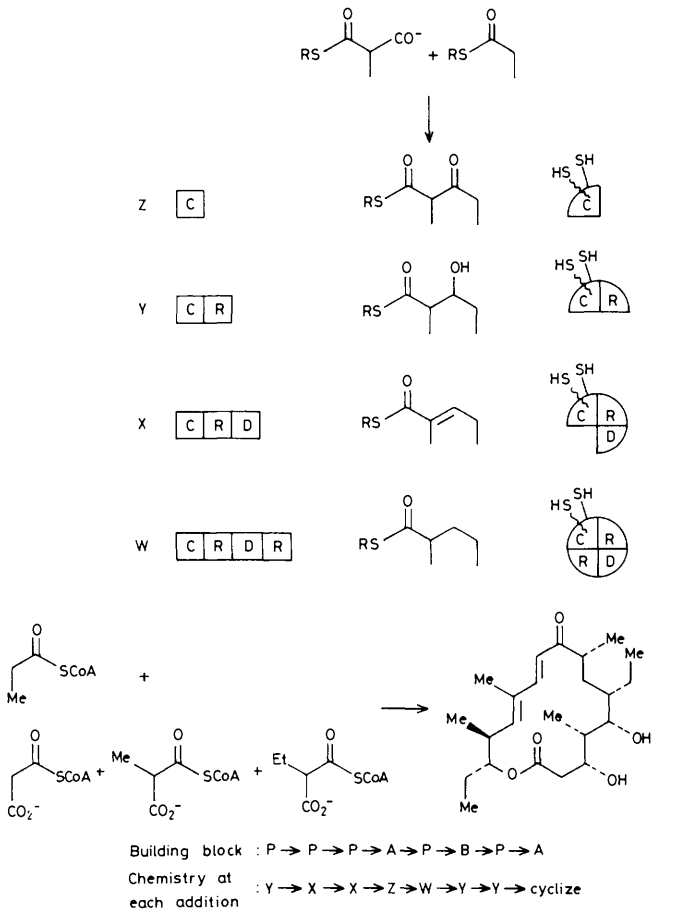
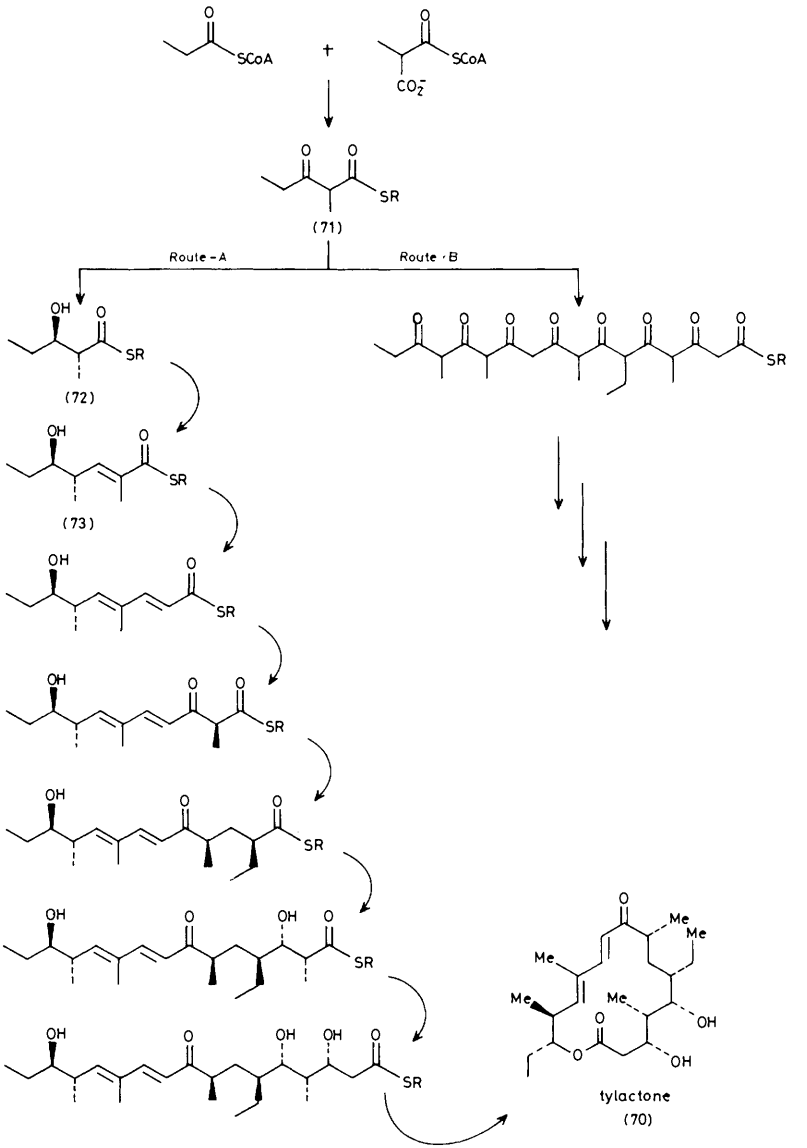


Figure 5 *Activities needed for tylactone formation*

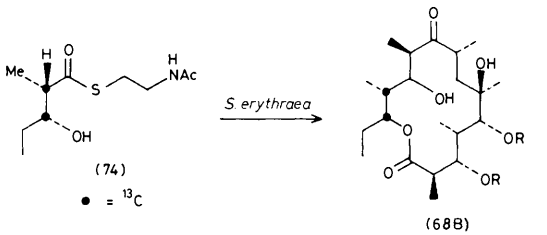
organism.[159] The use of a thioester in this way presumably allows the precursor to be loaded onto the PKS without requiring the co-operation of other *in vivo* fatty acid activating enzymes.[160] At the same time, some degradation of these materials to primary precursors appeared to occur, whereas a significant proportion was incorporated intact. With the very important *caveat* that these intact incorporations do not occur after the *in vivo* conversion of the administered materials into a poly-β-ketide, attached to the PKS, then these observations support the operation of the processive strategy of assembly (Route A, Scheme 18). A formalism that is helpful in charting the activities needed for tylactone formation is shown in Figure 5. Here the

[160] Compare; F. Lynen, *Fed. Proc.*, 1961, **20**, 941.



Scheme 18

synthesis is dissected into the constituent rounds of chain extension, which require one of the following; **Z**—a condensing enzyme only; **Y**—a condensation and reduction (affording an alcohol); **X**—a condensation, a reduction, and a dehydration (affording an alkene); **W**—a condensation, reduction, dehydration,



Scheme 19

and a final reduction (giving a saturated unit). The tylactone synthetase PKS should then catalyse a sequence of steps denoted by **YXXZWYY**, corresponding to the incorporation of PPPAPBPA building blocks, and conclude with macrolide ring formation.

Similar observations[161] have been made in studies of erythromycin-B (68B) and nonactin (75) formation. Thus, (74) can be incorporated into (68B) in whole-cell cultures of *S. erythraea* (Scheme 19), without being degraded into propionate units. Nonactin (75) is an interesting ionophore antibiotic, being composed of an alternating sequence of (+) and (−) nonactic acids (76) linked through ester bonds to afford a macrotetrolide.[162] The nonactic acids are unusual polyketides, in being derived from acetate, propionate, and succinate building blocks[163] according to the pattern shown in Scheme 20; it is not yet clear which is the starter unit,[164] nor how these units are linked together. However, the enantiomeric diols (77) and (78) have been synthesized as caprylcysteamine thiol esters, and in cultures of *S. griseus* they are efficiently and stereospecifically incorporated into nonactin, lending support to the notion that they are closely related to the *in vivo* products of the nonactin PKS system.[165] These encouraging results indicate that it may be possible to explore the various stages of macrolide and polyether assembly by this simple chemical approach, using whole-cell systems, as an aid to the task of devising specific assays, for components of the various PKSs, in cell-free systems.

Another observation which strengthens the analogy between the mode of action of FAS and these PKSs, is the inhibition of both by the antibiotic cerulenin (79). Cerulenin specifically and irreversibly inhibits the condensing enzyme[166,167] of FAS, by alkylating a peripheral cysteine residue in the active site.[168] It also inhibits the formation of 6-methylsalicylic acid catalysed by 6-

[161] D. E. Cane and C. C. Yang, *J. Am. Chem. Soc.*, 1987, **109**, 1255.
[162] W. Keller-Schierlein and H. Gerlach, *Fortschr. Chem. Org. Naturst.*, 1968, **26**, 161.
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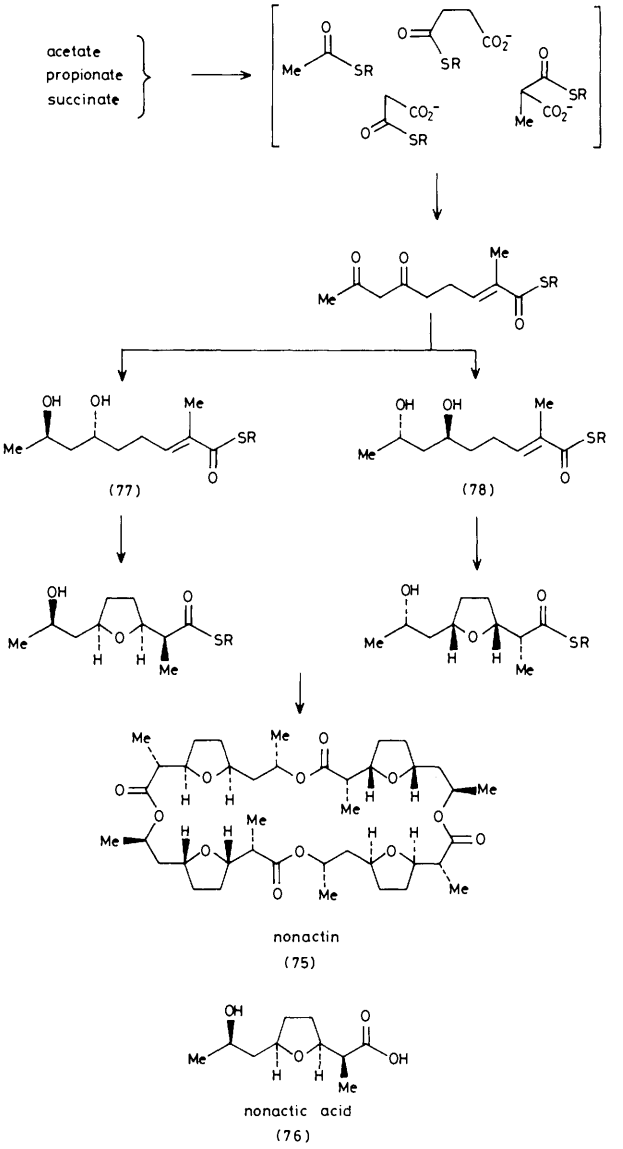
[164] C. A. Clark and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1985, 1568.
[165] Z. M. Spavold and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1988, 4.
[166] G. D. Agnolo, I. S. Rosenfeld, J. Awaya, S. Omura, and P. R. Vagelos, *Biochim. Biophys. Acta.*, 1973, **326**, 155.
[167] D. Vance, I. Goldberg, O. Mitsuhashi, K. Bloch, S. Omura, and S. Nomura, *Biochem. Biophys. Res. Commun.*, 1972, **48**, 649.
[168] A. Kawaguchi, H. Tomoda, S. Nozoe, S. Omura, and S. Okuda, *J. Biochem. (Tokyo)*, 1982, **92**, 7.


acetate
propionate
succinate
(77)
(78)
nonactin
(75)
nonactic acid
(76)

Scheme 20

methylsalicylic acid synthase[169] (*vide infra*), and when added to erythromycin-,[170] nonactin- and tylosin-[171,172] producing organisms, causes a cessation of antibiotic formation, implying that here too chain elongation is being blocked by the same (or a similar) mechanism. Building upon this analogy, Leadley and co-workers[170,173] prepared [^{3}H]tetrahydrocerulenin, and demonstrated that this reduced derivative not only inhibited mammalian FAS, but also when incubated with a cell-free extract of *S. erythraea*, it specifically labelled a protein of M_r about 40 000. The protein tagged by this [^{3}H]tetrahydrocerulenin, clearly, may be the condensing enzyme of the *S. erythraea* FAS, or the condensing activity of the erythronolide PKS, or both of these. The structure of this FAS, therefore, is of great interest, and extracts of *S. erythraea* were shown recently[174] to contain a small distinct polypeptide, identified as an acyl carrier protein, necessary for the production of fatty acids in extracts of *S. erythraea*. These data provide good evidence that the FAS in *S. erythraea* and Streptomycetes[175] more closely resembles the dissociable complex typical of *E. coli* than those seen in higher organisms. Thus the 3-oxoacyl synthase from *E. coli*[176] has a M_r of about 40 000, whereas the condensing enzyme of flavanone synthase from parsley[177] has a subunit M_r of 42 000 (*vide infra*). Whether or not the condensing enzyme from the erythronolide PKS also falls into this pattern remains an intriguing question.

Interestingly, the novel analogues 2-norerythromycins A–D (80) were isolated recently[178] from a genetically manipulated actinomycete. These must arise by the incorporation of an acetate unit into the chain, instead of a propionate unit, during the last round of elongation (*vide infra*).

(ii) *Post-assembly Transformations.* The steps between tylactone and tylosin have been mapped in detail by two groups, with the aid of blocked mutants,[179] and with the use of cerulenin[172] to block the early macrolide assembly processes. In these ways each intermediate could be isolated and its position in the pathway (Figure 6) could be determined by co-synthesis and bioconversion experiments. Once the individual steps had been identified, assays for specific enzymes could be determined in cell-free systems. Of particular interest is the observation of two hydroxylase activities, one acting at C-20 and the other at C-23. Both appear to

[169] H. Ohno, T. Ohno, J. Awaya, and S. Omura, *J. Biochem. (Tokyo)*, 1975, **78**, 1149.
[170] G. Roberts and P. F. Leadley, *FEBS Lett.*, 1983, **159**, 13.
[171] S. Omura, C. Kitao, J. Miyazawa, H. Imai, and H. Takashima, *J. Antibiot.*, 1978, **31**, 254.
[172] S. Omura, N. Sadakane, and H. Matsubara, *Chem. Pharm. Bull.*, 1982, **30**, 223.
[173] G. Roberts and P. F. Leadley, *Biochem. Soc. Trans.*, 1984, **12**, 642; *ibid.*, 1986, **14**, 568.
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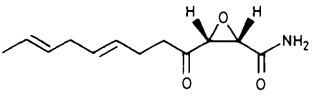
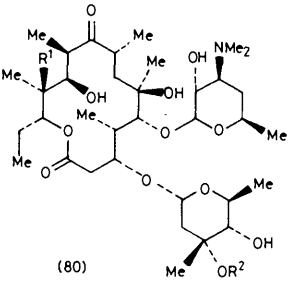
[178] J. B. McAlpine, J. S. Tuan, D. P. Brown, K. D. Grebner, D. N. Whitten, A. Buko, and L. Katz, *J. Antibiot.*, 1987, **40**, 1115.
[179] R. H. Baltz, E. T. Seno, J. Stonesifer, and G. M. Wild, *J. Antibiot.*, 1983, **36**, 131.



cerulenin
(79)



(80)

2- Norerythromycin-A R^1 = OH R^2 = Me
2- Norerythromycin-B R^1 = H R^2 = Me
2- Norerythromycin-C R^1 = OH R^2 = H
2- Norerythromycin-D R^1 = R^2 = H

be α-ketoglutarate and Fe^{2+} dependent dioxygenases,[180,181] with the turnover of substrate linked to the release of $[^{14}C]CO_2$ from [1-^{14}C]α-ketoglutarate.

The last step in the tylosin pathway is catalysed by macrocin *O*-methyltransferase. This enzyme is responsible for *O*-methylation of the 6-deoxy-D-allose moiety at the 3″-position. A sensitive assay for the enzyme was available, based upon the incorporation of radioactivity from [^{14}C-methyl]-*S*-adenosylmethionine,[182] or in conjunction with h.p.l.c.[183a] More recently, the protein has been purified to homogeneity[183b] and its N-terminal amino acid sequence determined.[184] Based upon these data, genes encoding parts of the tylosin pathway have now been cloned.

The pathway to the erythromycins has been studied in detail over several decades.[185] Following the formation of 6-deoxyerythronolide-B by the PKS, a soluble 6-deoxyerythronolide B hydroxylase[186] catalyses hydroxylation at C-6 to give erythronolide-B [(81) Scheme 21]. This hydroxylase has been purified to homogeneity,[187] it is a cytochrome-P450-type monooxygenase and appears in two forms which are separable by hydroxyapatite chromatography. Both cytochromes have an M_r of 44 220, and an identical N-terminal sequence for the first five amino acids. The cytochromes require two types of electron transport

[180] S. Omura, H. Tanaka, and M. Tsukui, *Biochem. Biophys. Res. Commun.*, 1982, **107**, 554.
[181] S. Omura, H. Tomoda, S. Yamamoto, M. Tsukui, and H. Tanaka, *Biochem. Biophys. Acta*, 1984, **802**, 141.
[182] E. T. Seno and R. H. Baltz, *Antimicrob. Agents Chemother.*, 1981, **20**, 370; *ibid.*, 1982, **21**, 758.
[183] (*a*) W. K. Yeh, N. J. Bauer, and J. E. Dotzlaf, *J. Chromatogr.*, 1984, **288**, 157. (*b*) N. J. Bauer *et al.*, *J. Biol. Chem.*, 1988, **263**, 15619 & 15626.
[184] S. E. Fishman, K. Cox, J. L. Larson, P. A. Reynolds, E. T. Seno, W. K. Yeh, R. Van Frank, and C. L. Hershberger, *Proc. Nat. Acad. Sci. USA*, 1987, **84**, 8248.
[185] For a review see: 'Antibiotics Vol. IV, Biosynthesis', ed. J. W. Corcoran, Springer-Verlag, New York, 1981.
[186] J. W. Corcoran and A. M. Vygantas, *Biochemistry*, 1982, **21**, 263.
[187] A. Shafiee and C. R. Hutchinson, *Biochemistry*, 1987, **26**, 6204.

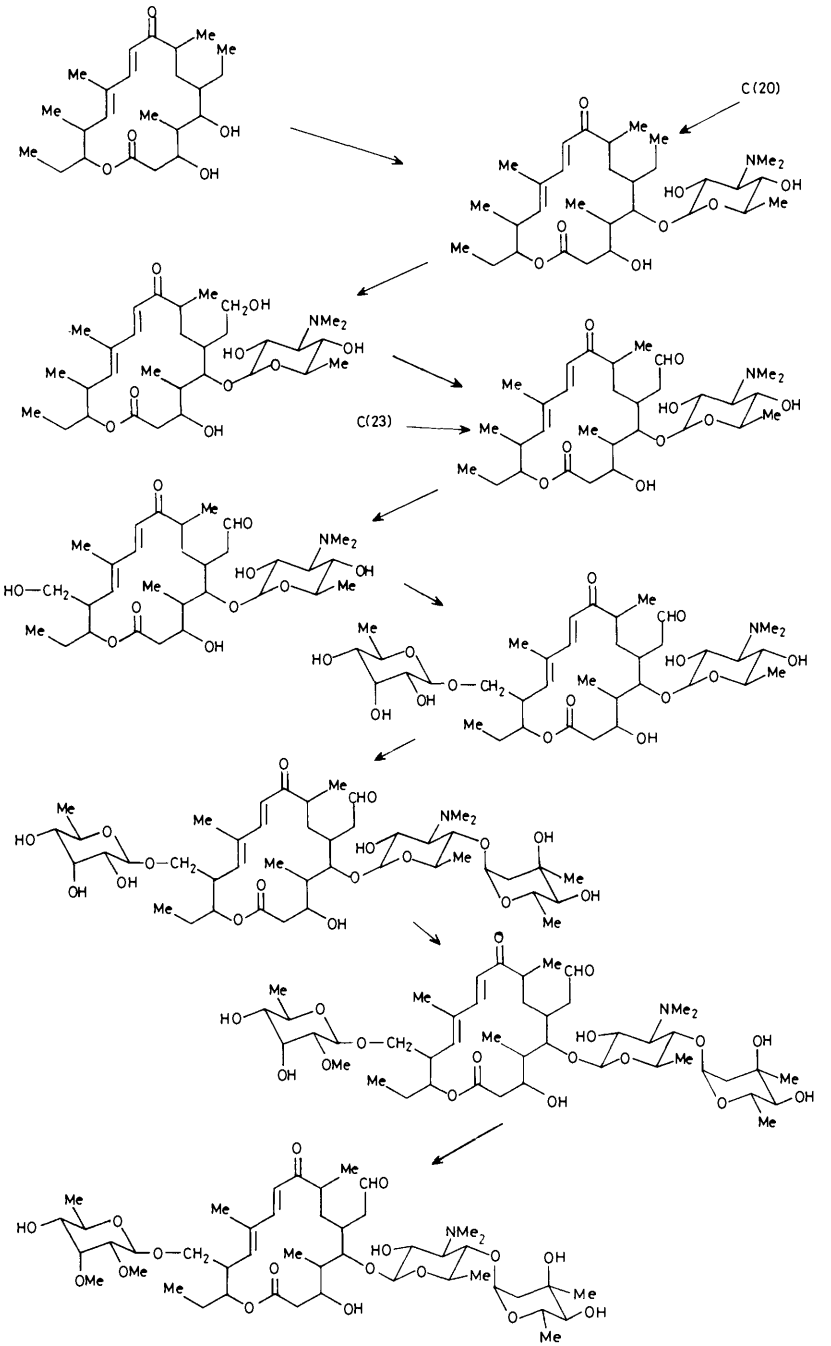
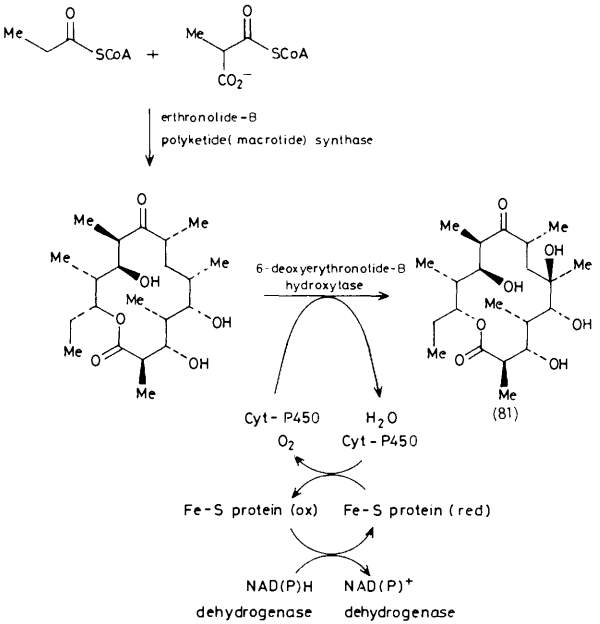


Figure 6 *The proposed pathway from tylactone to tylosin. The compounds which appear in this sequence are tylactone* → *O-mycaminosyltylactone* → *20-dihydro-23-deoxy-O-mycaminosyltylonolide* → *23-deoxy-O-mycaminosyltylonolide* → *O-mycaminosyltylonolide* → *demethyllactenocin* → *demethylmacrocin* → *macrocin* → *tylosin*

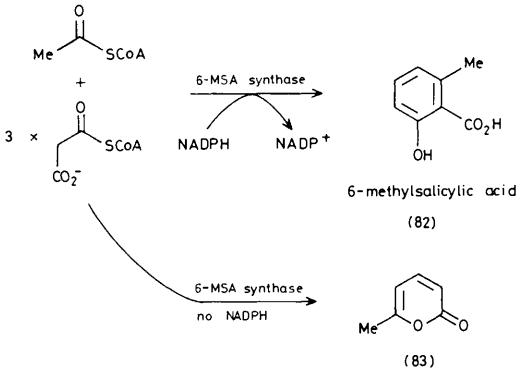


Scheme 21

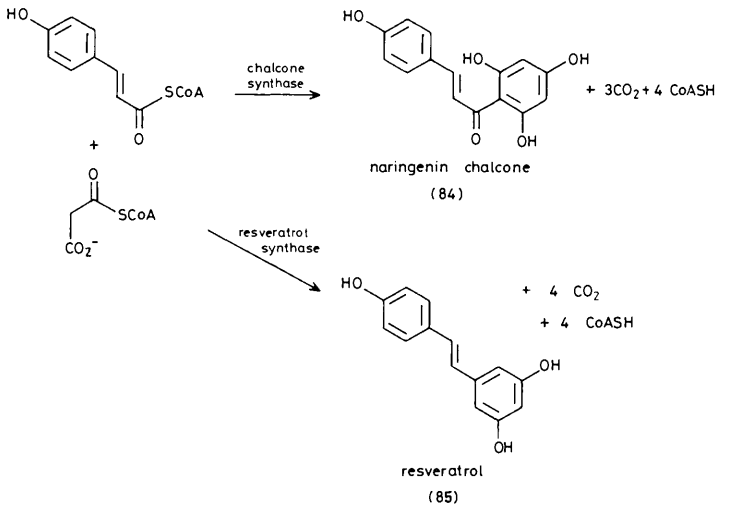
protein for activity.[188] One of these is an iron–sulphur protein, M_r 27 500, whose first twelve N-terminal amino acid residues are identical to those of the ferredoxin from *Mycobacterium smegmatis*. In addition, two NAD(P)H-dependent flavoproteins (FAD) were isolated, one of M_r 47 000 (dependent on NADPH), and the other with M_r 53 000 (dependent on NADH). It is not clear why the organism makes two different reductases, both of which support the hydroxylation of 6-deoxyerythronolide-B. This question may be addressed by future biochemical and genetic studies. Crude cell-free systems have also been developed that contain the C-12 hydroxylase[185] and C-3″-*O*-methyltransferase activities,[189] although neither has so far been purified to homogeneity.

B. Aromatic Polyketides.—One interesting aromatic polyketide synthase has been known for some time, namely the 6-methylsalicylic acid synthase[190] (Scheme 22) from *Penicillium patulum*. This enzyme catalyses the formation of 6-methylsalicylic (82) acid from an acetyl-CoA starter unit and three malonyl-CoAs, and requires NADPH. In the absence of this co-enzyme, the enzyme will instead catalyse the formation of triacetic acid lactone (83) from acetyl-CoA and

[188] A. Shafiee and C. R. Hutchinson, *J. Bacteriol.*, 1988, **170**, 1548.
[189] J. W. Corcoran, *Methods Enzymol.*, 1975, **43**, 487.
[190] P. Dimroth, E. Ringelman, and F. Lynen, *Eur. J. Biochem.*, 1976, **68**, 591.



Scheme 22



Scheme 23

two molecules of malonyl-CoA.[191] It appears that in the normal catalytic cycle, the reduction step with NADPH occurs before the final condensation with malonyl-CoA. The enzyme has an M_r of 1.1—1.5 × 10^6, and is very similar to the FAS in *P. patulum*;[190,192] both also contain a 4′-phosphopantetheine covalently attached to the protein.[193] This similarity is strengthened by the observation that both contain an acetyl transferase activity, and when treated with iodoacetamide both show a malonyl-CoA decarboxylase activity.[190] The structure of the 6-methylsalicylic acid synthase has not yet been described.

[191] P. Dimroth, E. Ringelman, and F. Lynen, *Eur. J. Biochem.*, 1970, **13**, 98.
[192] K. Holtermuller, E. Ringelman, and F. Lynen, *Hoppe-Seylers Z. Physiol. Chem.*, 1970, **351**, 1411.
[193] P. Dimroth, G. Gruell, R. Seyffert, and F. Lynen, *Hoppe-Seylers Z. Physiol. Chem.*, 1972, **353**, 126.

Although not strictly within the scope of this review, it is of interest to note that both chalcone synthase[194–203] (CHS), and stilbene (resveratrole) synthase[204] (RS) have been purified from various plant species (Scheme 23). Chalcone synthase catalyses the formation of naringenin chalcone (84) using 4-coumaryl-CoA, as a starter unit, and three molecules of malonyl-CoA. The reaction requires no reduction step, and in contrast to both the 6-methylsalicylic acid synthase, and FAS, the chalcone synthase[194] is typically a relatively small protein, M_r 84 000, being a dimer of identical subunits, each of about M_r 42 000. The enzyme also *does not* contain a 4′-pantetheinyl residue. It has, however, been cloned[205–209] and the gene sequenced and compared to the sequence of the β-ketoacyl-ACP synthase of FAS. The substrate specificity of the *Petroselinum hortense* enzyme has been explored.[198] Butyryl-CoA, hexanoyl-CoA, and benzoyl-CoA all served as substrates in the condensation, with similar efficiency to 4-coumaryl-CoA.

Resveratrole synthase[204] is a key enzyme in the biosynthesis of stilbene-type phytoalexins, and catalyses the formation of resveratrol (85), again from 4-coumaryl-CoA and three molecules of malonyl-CoA. The enzyme is closely similar to the CHS; it is a dimer of identical subunits M_r 42 700 and does not contain 4′-phosphopantetheine. The genomic and cDNA for the enzyme has been cloned and sequenced.[210] This has revealed a high homology with the gene for CHS throughout the coding region, with an intron at the same position in genomic DNA as an intron that is conserved in all chalcone synthase genes. However, a substantial number of amino acid differences to CHS occur in positions highly conserved in all CHS enzymes. It has been proposed[210] that the two proteins possess a common scaffold necessary for binding the substrates and for catalysis, and that the differences, amounting to 15.9% of the 270 amino acids highly conserved in all CHS enzymes, are responsible for the formation of different products. The differences are not confined to a specific part of the protein, but appear to be scattered throughout the amino acid sequence.

[194] F. Kreuzaler, H. Ragg, W. Heller, R. Tesch, I. Witt, D. Hammer, and K. Hahlbrock, *Eur. J. Biochem.*, 1979, **99**, 89.
[195] W. Heller and K. Hahlbrock, *Arch. Biochim. Biophys.*, 1980, **200**, 617.
[196] R. Suetfeld and R. Wiermann, *Arch. Biochim. Biophys.*, 1980, **201**, 64.
[197] R. Suetfeld and R. Wiermann, *Z. Naturforsch. Teil C*, 1981, **36**, 30.
[198] R. Schuez, W. Heller, and K. Hahlbrock, *J. Biol. Chem.*, 1983, **258**, 6730.
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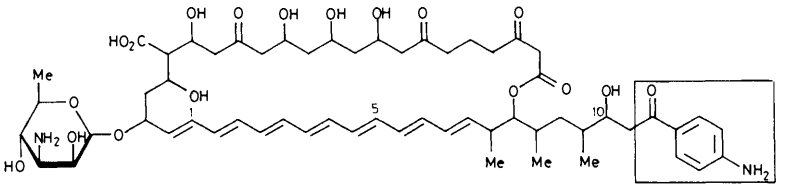
[200] L. Beerhues and R. Wiermann, *Z. Naturforsch. Teil C*, 1985, **40**, 160.
[201] W. Hinderer and H. U. Seitz, *Arch. Biochim. Biophys.*, 1985, **240**, 265.
[202] Y. Ozeki, K. Sakano, A. Komamine, Y. Tanaka, H. Noguchi, U. Sankawa, and T. Suzuki, *J. Biochem. (Tokyo)*, 1985, **98**, 9.
[203] G. Hrazdina, E. Lifsen, and N. F. Weeden, *Arch. Biochim. Biophys.*, 1986, **247**, 414.
[204] A. Schoppner and H. Kindl, *J. Biol. Chem.*, 1984, **259**, 6806.
[205] U. Reimold, M. Kroeger, F. Kreuzaler, and K. Hahlbrock, *EMBO J.*, 1983, **2**, 1801.
[206] H. Reif, U. Niesbach, D. Deumling, and H. Saedler, *Mol. Gen. Genet.*, 1985, **199**, 208.
[207] H. Sommer and H. Saedler, *Mol. Gen. Genet.*, 1986, **202**, 429.
[208] U. Wienand, U. Weydemann, U. Niesbach-Kloesgen, P. A. Peterson, and H. Saedler, *Mol. Gen. Genet.*, 1986, **203**, 202.
[209] R. E. Koes, C. E. Spelt, J. N. M. Mol, and A. G. M. Gerats, *Plant Mol. Biol.*, 1987, **10**, 159.
[210] G. Schroder, J. W. S. Brown, and J. Schroder, *Eur. J. Biochem.*, 1988, **172**, 161.

5 The Molecular Biological Approach to Antibiotic Biosynthesis in Streptomycetes

The rapid progress in molecular genetic studies of *Streptomyces*, now underway, is affording basic information on the organization and regulation of genes concerned with antibiotic biosynthesis in these organisms. An important feature, in both *Streptomyces* and *Bacillus*, as compared to the enteric bacteria and higher organisms, is the high frequency of clustered and overlapping genes[211,212] encoding the enzymes and regulatory proteins of individual pathways. This greatly facilitates the isolation and characterization of all the biosynthetic genes on a pathway, once a strategy is available to identify just one. In the case of antibiotic production, a resistance gene must also be expressed, in order to protect the host cell from the biological effects of its own antibiotics, and this also is usually closely linked to the biosynthetic structural genes.

There are several strategies available for cloning the genes of antibiotic biosynthesis in *Streptomyces*, these have been elaborated previously by Hopwood,[213] and are listed in Table 9. The following discussion describes these strategies, and the successes that have been achieved recently using these approaches in studies of antibiotic biosynthesis. In the future, it will almost certainly be through a unison of the chemical, biochemical, and molecular genetical approaches that the most exciting advances will be made in this area.

A. Candicidin and Actinomycin.—Candicidin (86) is a member of the heptaene family of polyene macrolide antibiotics. The construction of its carbon backbone appears to require a PKS that uses *p*-aminobenzoyl-CoA as a starter unit.[214]



Candicidin
(86)

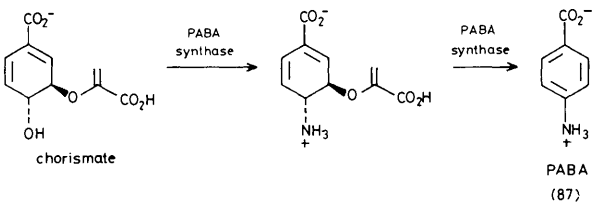
The *p*-aminobenzoic acid (87) arises from chorismic acid, in an interesting reaction catalysed by *p*-aminobenzoic acid synthase (PABA synthase) (Scheme 24). The enzyme has been partially purified from a recombinant *E. coli*[215] and from *S. griseus* IMRU3570,[214] and by gel-filtration it has M_r 50 000. There is a

[211] D. A. Hopwood, in 'Biochemistry and Genetic Regulation of Commercially Important Antibiotics', ed. L. C. Vining, Addison Wesley, 1983.
[212] H. Zalkin and D. J. Ebbole, *J. Biol. Chem.*, 1988, **263**, 1595.
[213] D. A. Hopwood, in Proceedings Int. Symp. Biol. Actinomycetes, 1986; see also J. T. Feyerman, *Biotechnology*, 1986, **4**, 786.
[214] J. F. Martin, in 'Biochemistry and Genetic Manipulation of Commercially Important Antibiotics', ed. L. C. Vining, Addison Wesley, London, 1983.
[215] C. T. Walsh, M. D. Erion, A. E. Watts, J. J. Delaney, and G. A. Berchtold, *Biochemistry*, 1987, **26**, 4734.

Table 9 *Some Strategies for Cloning* Streptomyces *Antibiotic Biosynthetic Genes (ref.* 213)

Strategy	*Antibiotic*	*Reference*
1. Detection of an individual gene product through cloning and expression in a standard host	Candicidin (PABA synthase)	216
	Actinomycin (Phenoxazinone synthase)	120
2. Complementation of blocked mutants in the producing strain	Undecylprodigiosin	222, 223
	Actinorhodin	227
	Clavulanic Acid	81
	Streptomycin	263
	Tetracenomycin	231
	Desferrioxamine-B	264
3. Mutational cloning in the producing strain	Methylenomycin	255, 260
4. Cloning of antibiotic resistance in a standard host or in a sensitive mutant, followed by analysis of linked DNA for biosynthetic genes	Oxytetracycline	237
	Bialaphos	244—246
	Erythromycin	249, 250
	Puromycin	253
5. Probing gene libraries with synthetic oligonucleotide probes designed from amino acid sequences of biosynthetic enzymes	Penicillin	24
	Tylosin	184
6. Direct cloning of large DNA fragments from the antibiotic producer into a convenient non-producing host, and screening for antibiotic production	Cephamycin-C	265
7. Use of cloned DNA for one antibiotic to detect homologous genes for a related antibiotic	Granaticin	262
	Milbemycin	262

close correlation between candicidin productivity and the activity of PABA synthase in phosphate repressed or derepressed wild-type cultures and in mutants partially blocked in candicidin production.[214] It seems clear, therefore, that PABA synthase is involved in candicidin biosynthesis. *p*-Aminobenzoic acid is also a key constituent of folic acid, an essential vitamin for the normal vegetative growth of this (and other) organism(s), and the cloning strategy took advantage of this relationship. Thus *Bam* H1 fragments of *S. griseus* genomic DNA, and from a sulphonamide-resistant mutant of the strain (this resistance to sul-



Scheme 24

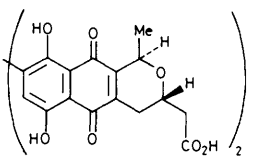
phonamide apparently arises due to overproduction of PABA synthase), were inserted[216] into the plasmid pIJ41, and used to transform *S. lividans 66*. One of the thiostreptone-resistant transformants was subsequently isolated because it too had now inherited a sulphonamide-resistant phenotype. The cloned DNA in this transformant also restored prototrophy to a *pab* auxotroph of *S. lividans*, and must therefore contain a structural gene for the enzyme. It is not clear whether the same or different forms of PABA synthase are involved in folic acid biosynthesis and candicidin biosynthesis, although Southern hybridization analysis revealed a single band in *S. griseus* DNA that had strong homology with the cloned *pab* fragment, indicating the presence of only a single form of the structural gene for this enzyme.

As described earlier, phenoxazinone synthase catalyses the formation of the phenoxazinone ring in the actinomycins (Scheme 13). A structural gene for this enzyme was cloned[120] from *S. antibioticus* by inserting *Sph* I fragments of *S. antibioticus* genomic DNA into the vector pIJ702, the recombinant plasmids transformed *S. lividans* to the thiostreptone-resistant phenotype, and batches of these transformants were then assayed for the enzyme activity. Eventually a single transformant was selected which showed phenoxazinone synthase activity, with 3-hydroxyanthranilate as substrate in a cell-free extract, and this contained a plasmid with a single 2.45 kb insert containing the gene for the 88 000 kDa subunit of the enzyme.

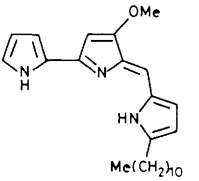
B. Complementation of Blocked Mutants.—Introducing a block at the DNA level in a multistep biosynthetic pathway, by causing damage to one or more of the structural genes, will prevent the synthesis of the end product due to loss of one or several of the essential enzymic activities. Blocked mutants may, however, be restored to their original phenotype if extrachromosomal DNA on a suitable vector can be introduced and stably maintained, containing a good copy of the damaged DNA. The mutation carried by the organism will then be complemented, and in the case of antibiotic biosynthesis, antibiotic production will be restored, a phenotype that is usually easy to assay. The method is best applied to organisms that are genetically well defined, *i.e.* for which a genetic map exists, and most importantly, for which relevant mutants are available. Also, plasmid or phage vectors must stably maintain the cloned DNA of interest in the host strain, a requirement that is by no means easily met with some strains of *Streptomyces*.

Streptomyces coelicolor A3(2), genetically the best studied Streptomycete,[217] has chromosomally coded pathways for the biosynthesis of two chemically distinct pigmented antibiotics, actinorhodin[218] (88) a red-blue pH indicator, and a red pigment undecylprodigiosin[219] (89). Analysis of mutants blocked in each pathway (*act* and *red* mutants, respectively), by co-synthesis tests and by genetic

[216] J. A. Gil and D. A. Hopwood, *Gene*, 1983, **25**, 119.
[217] D. A. Hopwood, *Bacteriol. Rev.*, 1967, **31**. 373.
[218] H. Brockman, A. Zeeck, K. van der Merve, and W. Muller, *J. Lieb. Annal. Chem.*, 1966, **698**, 3575.
[219] See: H. H. Wasserman, C. K. Shaw, R. J. Sykes, and R. J. Cushley, *Tetrahedron Lett.*, 1974, 2787.



actinorhodin
(88)



undecylprodigiosin
(89)

mapping, led to the recognition of a separate cluster of genes coding for each pathway.[220,221] Since the two metabolites were pigments, the assay for complementation was experimentally very convenient.

In the case of undecylprodigiosin, mutants were identified by co-synthesis and enzyme assay that were blocked specifically in the *O*-methylation step[222,223] requiring SAM. One such mutant was the host for *Bcl* I digested *S. coelicolor* genomic DNA, inserted into the *Bgl* II site of pIJ702. After transformation one of the resulting colonies had the Red^+ phenotype, typical of undecylprodigiosin production. This contained a plasmid having a 4.7 kb insert, part of which was associated with the pathway. Subsequently, other clones were isolated from the flanking DNA, which complemented mutants blocked at other positions in the pathway, thus providing physical evidence to confirm the conclusions from genetic mapping, that all the biosynthetic genes are clustered on the chromosome.

The best studied pathway from a genetic viewpoint is that leading to the isochromanequinone antibiotic actinorhodin (88). In pioneering work, Rudd and Hopwood[220] isolated 75 mutants of *S. coelicolor* blocked in the biosynthesis of this antibiotic, and were able to group these into seven phenotypic classes. The classification was based on the mutants' ability to secrete intermediates which other mutants, blocked earlier in the pathway, could assimilate and convert into actinorhodin, when grown together in culture (the so-called co-synthesis test). The results are given in Table 10, and lead initially to seven classes of *act* mutants, which apart from those of class II that appear to define a positively acting control gene, identify at least seven biosynthetic genes acting in the following sequence:

$$\text{acetate} \xrightarrow{\text{I, III}} \xrightarrow{\text{VII}} \xrightarrow{\text{IV}} \xrightarrow{\text{VI}} \xrightarrow{\text{Va, Vb}} \text{actinorhodin}$$

Actinorhodin is a polyketide whose carbon skeleton is derived from eight intact acetate units, one acetyl-CoA starter unit, and seven malonyl-CoA extender units. The *act* I and *act* III mutants fail to secrete any biosynthetic intermediate

[220] B. A. M. Rudd and D. A. Hopwood, *J. Gen. Microbiol.*, 1979, **114**, 35.
[221] B. A. M. Rudd and D. A. Hopwood, *J. Gen. Microbiol.*, 1980, **119**, 333.
[222] J. S. Feitelson and D. A. Hopwood, *Mol. Gen. Genet.*, 1983, **190**, 394.
[223] J. S. Feitelson, F. Malpartida, and D. A. Hopwood, *J. Gen. Microbiol.*, 1985, **131**, 2431.

Table 10 *Classes of* act *mutants and their phenotype*

Mutant class	*Type strain*	*No. of mutants in class*	*Diffusable pigment*	*Co-synthesis*	
				as converter	*as secretor*
I	B78	13	—	IV, V, VI, VII	—
II	2377	26	—	—	—
III	B41	7	red	IV, V, VI, VII	—
VII	B40	2	light brown	IV, V, VI	I, III
IV	B17	5	reddish brown	V, VI	I, III
VI	B22	2	light brown	V	I, III, IV,
V	B1	21	brown	—	I, III, IV, VI

capable of conversion to actinorhodin by mutants of other classes, but convert intermediates secreted by all other mutants (except those of class II) to actinorhodin. Thus *act* I and *act* III mutants define the earliest steps in the pathway, and are obvious candidates for carrying mutations in genes coding for components of the actinorhodin PKS. The *act* III mutants accumulate a red pigment of unknown structure, which must be rationalized as a shunt product. Mutant classes IV, V, VI, and VII, by virtue of their ability to serve as secretors to other mutants, must produce diffusible intermediates of actinorhodin biosynthesis. Recently the isolation of some of these compounds has been reported,[224,225] and based on their structures a reasonable pathway to actinorhodin was proposed (see Scheme 25).

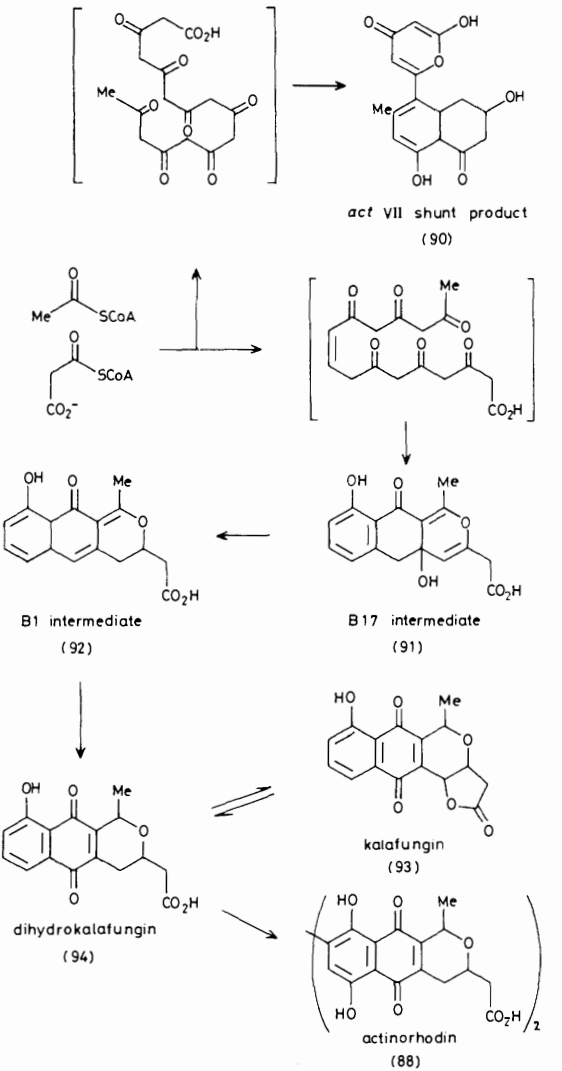
Analysis of extracts of mutant B40, a member of class VII, the earliest class of secretor mutants, revealed an intermediate that was active in co-synthesis assays, but unstable and refractory to purification. However, another compound was isolated from this class which showed no biological activity, and had the structure (90). This presumably represents a shunt product, because it must arise by a mode of polyketide folding and cyclization that is different to that necessary for the production of actinorhodin.

Class IV mutants, representing a block in the next indentified step in the pathway, accumulate a biosynthetic intermediate (91) with the correct ring structure, showing that the *act* IV gene must act at a step after chain assembly and ring formation. Mutant strain B22 of the next secretor class, class VI, also made a biologically active material that was colourless, unstable, and refractory to isolation. However, strain B1, a member of class V blocked late in the pathway, yielded an active material identified as (92). The conversion of (92) into actinorhodin still requires several steps, and other mutants in this class were also examined for further intermediates. One, mutant B135, was found to secrete two compounds, only one of which was identified as the antibiotic kalafungin (93), itself a known[226] metabolite of a different Streptomycete. The pathway may be

[224] H. G. Floss, S. P. Cole, Y. G. He, B. A. M. Rudd, J. Duncan, I. Fujii, C. Chang, and P. J. Keller, in 'Regulation of Secondary Metabolite Formation', ed. H. Kleinkauf, H. von Dohren, H. Dornauer, and G. Neseman, Hoechst Workshop Conference, Vol. 16, Verlagsgesellschaft, Weinheim, FRG, 1986.

[225] S. P. Cole, B. A. M. Rudd, D. A. Hopwood, C. Chang, and H. G. Floss, *J. Antibiot.*, 1987, **40**, 340.

[226] H. Hoeksema and W. C. Kreuger, *J. Antibiot.*, 1976, **29**, 704.



Scheme 25

completed by dimerization of (94), and hydroxylation of the aromatic A ring (or *vice versa*).

In order to clone this pathway, a mutant of *act* class V (and also carrying a *red* mutation to abolish the production of undecylprodigiosin), was selected as a recipient for cloned DNA from the wild type *act*$^+$ strain. *Mbo* I fragments of *S. coelicolor* genomic DNA, inserted into the unique *Bam* HI site in the plasmid

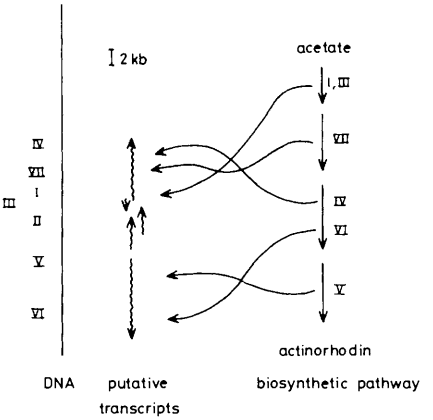


Figure 7 *Organization of the* act *cluster in* S. coelicolor A3(2) *showing location of mutant classes on the chromosomal DNA, the putative transcription units, and the positions of the* act *mutant classes in the biosynthetic pathway*

pIJ922, were used to transform the mutant.[227] Two blue colonies were detected amongst around 8000 transformants, the blue colour indicating actinorhodin production. The plasmids present in these transformants were isolated and shown to contain inserts of 34 kb and 16 kb, with an overlapping region of 12 kb. By splicing together these inserts, a new recombinant plasmid was prepared containing the central 32.5 kb portion, which could not only complement strongly *act* mutants of all seven classes, but when introduced into *S. parvalus*, a strain that does not produce actinorhodin, it conferred on the strain the ability to produce actinorhodin. This indicates that DNA encoding the entire actinorhodin pathway is carried by the plasmid. More recently[228] a full physical and genetic characterization of this gene cluster was reported. Complementation and mutational cloning analyses indicated that all the biosynthetic genes, including at least one regulatory gene and a resistance gene, are clustered in a chromosomal region of about 26 kb. The genes are organized in at least four separate transcription units, ranging from a small monocistronic transcript of 1 kb for the class III gene, to a polycistronic transcript of at least 5 kb for the class I, VII, and IV regions (Figure 7). The DNA sequence of the *act* III region has been determined,[229] and reveals an open reading frame whose translation product shows significant homology with certain oxidoreductases, lending support to the notion that this protein is at least the dehydrogenase required to reduce the polyketide chain at C-9 during chain assembly.

Following this cloning work, the first successful attempts were made[230] to generate novel hybrid antibiotics by transferring the actinorhodin biosynthetic

[227] F. Malpartida and D. A. Hopwood, *Nature*, 1984, **309**, 462.
[228] F. Malpartida and D. A. Hopwood, *Mol. Gen. Genet.*, 1986, **205**, 66.
[229] S. E. Hallam, F. Malpartida, and D. A. Hopwood, *Gene*, 1988, in press.
[230] D. A. Hopwood, F. Malpartida, H. M. Kieser, H. Ikeda, J. Duncan, I. Fujii, B. A. M. Rudd, H. G. Floss, and S. Omura, *Nature*, 1985, **314**, 642.

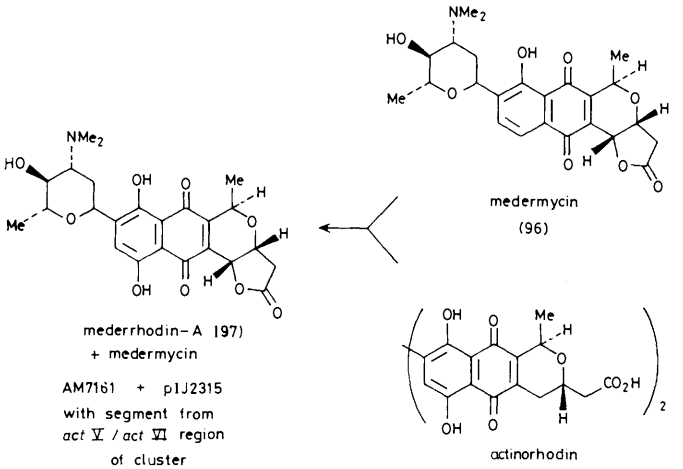


Figure 8 *Production of the hybrid compound mederrhodin-A by* Streptomyces *sp.* AM-7161 *carrying* pIJ2315

genes into other Streptomycetes. The new hosts were strains that produce different isochromanequinone antibiotics, granaticin (95) and medermycin (96). Actinorhodin, granaticin, and medermycin are all acid–base indicators which confer characteristic colours on the cultures producing them (red ⇌ blue for actinorhodin, red ⇌ purple for granaticin, and yellow ⇌ brown for medermycin). When plasmids containing regions of the *act* gene cluster were introduced into *Streptomyces* sp. AM-7161 (medermycin producer), *S. violaceoruber Tu22* (granaticin producer), and the mutant strain B140 (blocked in granaticin), the colours of some of the cultures arising clearly indicated that one or more novel compounds were present. Thus, AM-7161 carrying pIJ2315 (Figure 8) produced large amounts of the hybrid compound (97), called mederrhodin-A (bright purple at high pH), whereas *S. violaceoruber* carrying pIJ2303 (Figure 9) produced, in addition to actinorhodin, the hybrid (98) called dihydrograntirhodin (blue-purple). There seems little doubt that these novel products arise through the interplay of enzymes encoded by structural genes originating from different Streptomycetes.

A further example of this approach is the isolation recently[231] of a gene cluster for the biosynthesis of tetracenomycin-C (99) (Scheme 26) one of the important family of anthracycline antitumour antibiotics. In this case mutants of *S. glaucescens* blocked in tetracenomycin production[232,233] were complemented by DNA fragments derived from the wild strain, cloned in the high copy plasmid pIJ702. The cloned *tcm* DNA included the production and resistance genes, which again form a single cluster on the chromosome of *S. glaucescens.*

[231] H. Motamedi and C. R. Hutchinson, *Proc. Nat. Acad. Sci. USA*, 1987, **84**, 4445.
[232] H. Motamedi, E. Wendt-Pienkowski, and C. R. Hutchinson, *J. Bacteriol.*, 1986, **167**, 575.
[233] S. Yue, H. Motamedi, E. Wendt-Pienkowski, and C. R. Hutchinson, *J. Bacteriol.*, 1986, **167**, 581.

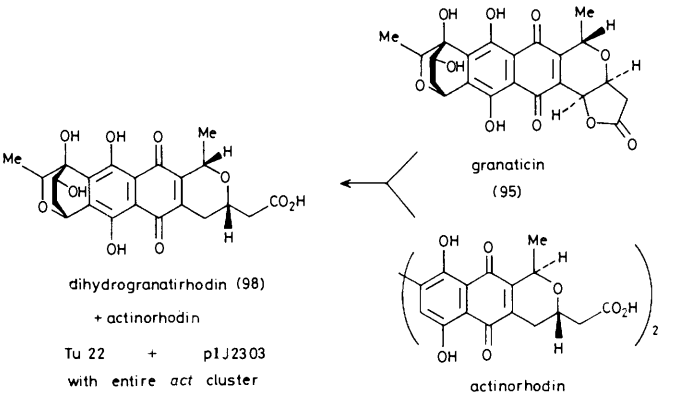
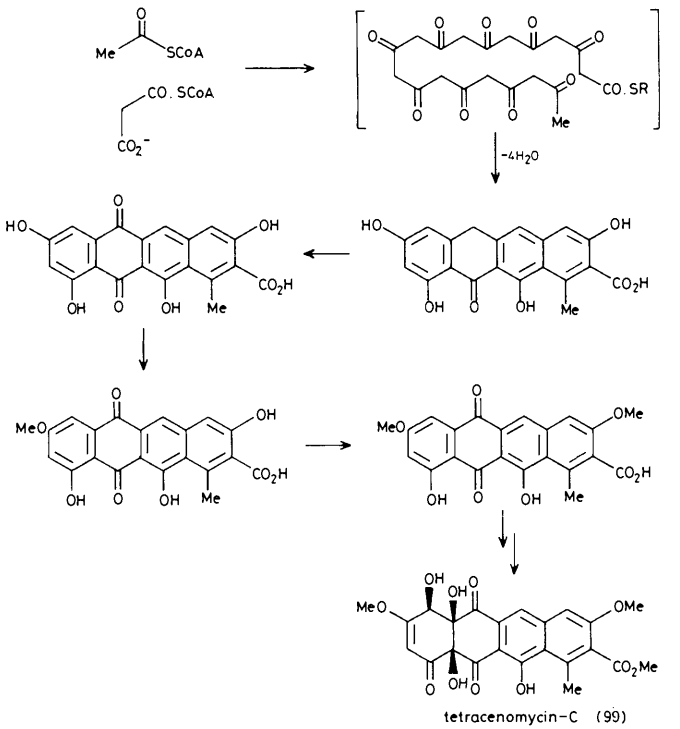


Figure 9 *Production of the hybrid compound dihydrograntirhodin by* S. violaceoruber *carrying* pIJ2303



Scheme 26

The same multicopy plasmid pIJ702 has been used to isolate DNA encoding parts of the clavulanic acid pathway, as described earlier.

C. Cloning of Antibiotic Resistance Genes.—It is often convenient to clone antibiotic resistance genes, since the screen for specific clones having an antibiotic resistant phenotype is usually straightforward. When the resistance gene(s) is (are) closely linked to the antibiotic biosynthetic genes, it is then in principle a simple matter to analyse the flanking DNA in order to locate the latter, as illustrated in the following examples.

The known pathway to oxytetracycline (100), an important broad spectrum antibiotic in clinical use, is shown in Scheme 27. Most of the intermediates shown were isolated from blocked mutants,[234,235] and recently the purification of one enzyme on the pathway was described,[236] a flavin-dependent anhydrotetracycline oxygenase from *S. hygroscopicus*. The oxytetracycline structural genes appear to be clustered on the chromosome of *S. rimosus* (contrary to earlier reports), and also in this cluster is at least one *otc* resistance gene. An *otc* resistance gene was cloned[237] from *S. rimosus* by inserting *Pst* I fragments of chromosomal DNA into plasmid pPZ12, and transforming an OTC-sensitive mutant to recover the OTC resistance phenotype. The resistance to OTC was shown to be associated with one 8.7 kb insert in the plasmid, and Southern hybridization revealed that this fragment was also present in *Pst* I cut *S. rimosus* chromosomal DNA, but not in DNA from the OTC-sensitive mutant. This fragment was then used to detect hybridizing clones in a lambda library in *E. coli*, which together provided some 30—40 kb of DNA surrounding the resistance gene, including some of the biosynthetic genes. The full details of the characterization of this DNA have not yet been published.

Bialaphos (101) is a tripeptide antibiotic containing two L-alanine residues and an analogue of L-glutamic acid called phosphonothricin (102). When it is released by peptidases, the phosphonothricin is a potent inhibitor of glutamine synthase. This fascinating pathway, parts of which have a striking resemblance to the citric acid cycle, has been elucidated largely by analysing non-producing mutants,[238–243] and the details are shown in Figure 10. A cloned bialaphos resistance gene, isolated from *S. hygroscopicus* DNA by selection in a bialaphos sensitive host (*S.*

[234] P. M. Rhodes, N. Winskill, E. J. Friend, and M. Warren, *J. Gen. Microbiol.*, 1981, **124**, 329.
[235] R. Thomas and D. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1983, 128 and 677.
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[239] A. Satoh, S. Inouye, T. Niida, and N. Otake, *J. Antibiot.*, 1983, **36**, 96.
[240] H. Seto, S. Imai, T. Tsuruoka, H. Ogawa, A. Satoh, T. Sasaki, and N. Otaki, *Biochem. Biophys. Res. Commun.*, 1983, **111**, 1008.
[241] H. Ogawa, A. Satoh, S. Inouye, T. Niida, and N. Otake, *J. Antibiot.*, 1984, **37**, 1509.
[242] S. Imai, H. Seto, T. Sasaki, T. Turuoka, H. Ogawa, A. Satoh, S. Inouye, T. Niida, and N. Otake, *J. Antibiot.*, 1984, **37**, 1505.
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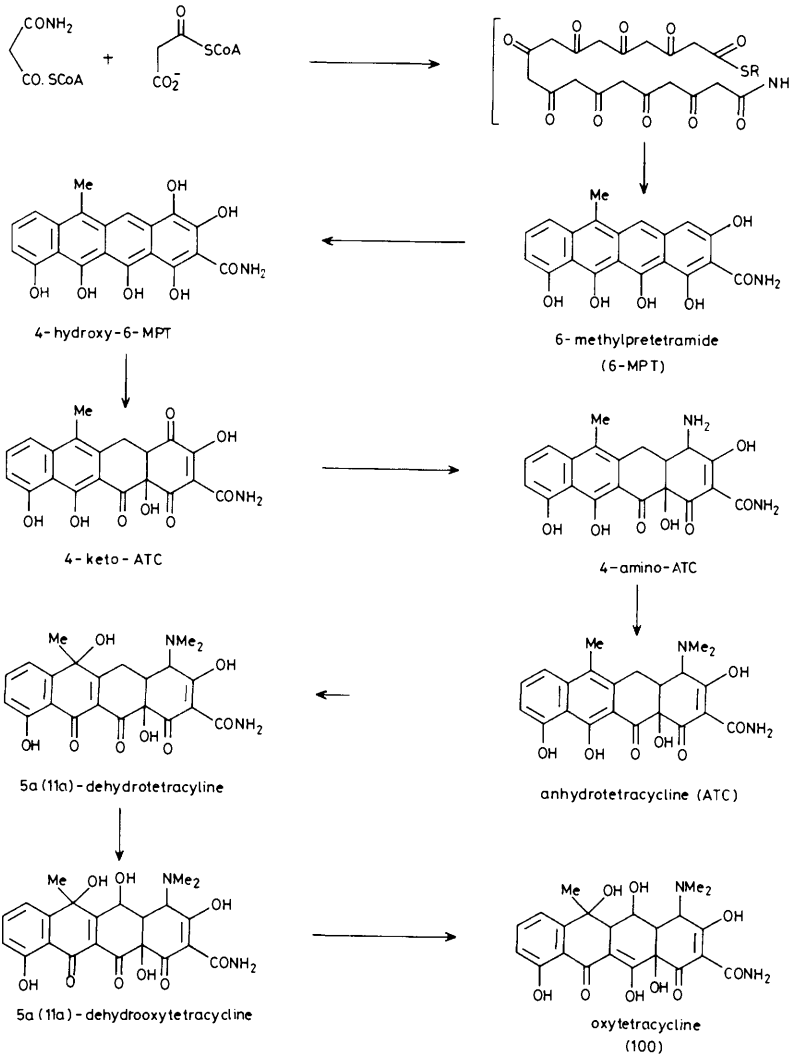
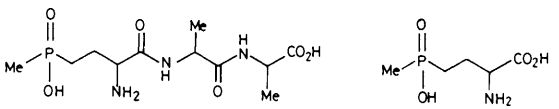

CONH2
CO. SCoA
+
SCoA
CO2−
SR
NH2
Me
OH
CONH2
4-hydroxy-6-MPT
6-methylpretetramide
(6-MPT)
4-keto-ATC
4-amino-ATC
NMe2
5a (11a)-dehydrotetracyline
anhydrotetracycline (ATC)
5a (11a)-dehydrooxytetracycline
oxytetracycline
(100)

Scheme 27


bialaphos
(101)
phosphonothricin
(102)

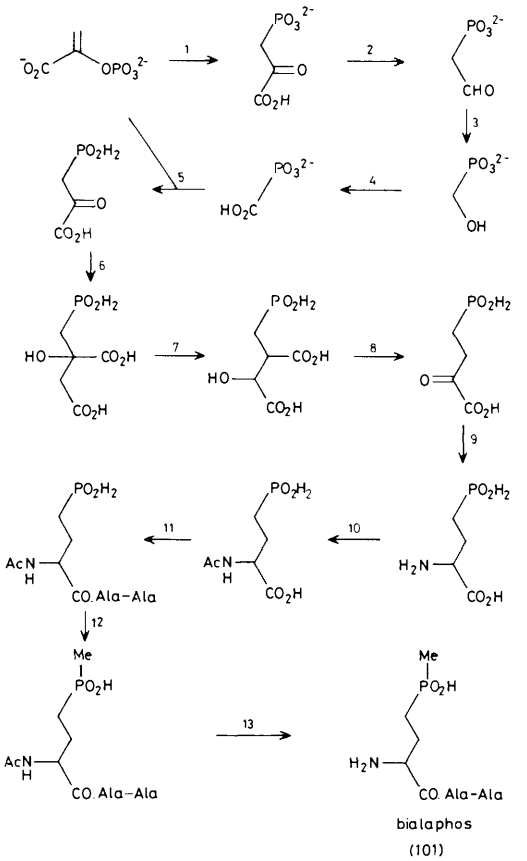


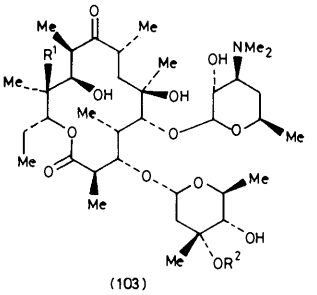
Figure 10 *Biosynthesis of bialaphos*

lividans), was used to detect hybridizing DNA in a cosmid library prepared in *E. coli*. DNA from these subclones was introduced into pIJ702, and the recombinant plasmids were then introduced into bialaphos non-producing mutants, where complementation occurred leading to bialaphos production.[244–246] Two of the subclones carried, respectively, four clustered genes for early biosynthetic steps, and four clustered genes for late steps, plus the resistance gene. Southern hybridization analysis showed that the two clusters were adjacent on the chromosome. However, the genes for other steps in the pathway remain to be

[244] T. Murakami, H. Anzai, S. Imai, A. Satoh, K. Nagaoka, and C. J. Thompson, *Mol. Gen. Genet.*, 1986, **205**, 42.

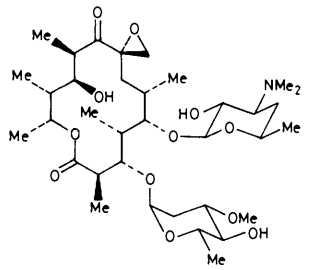
[245] H. Anzai, T. Murakami, S. Imai, A. Satoh, K. Nagaoka, and C. J. Thompson, *J. Bacteriol.*, 1987, **169**, 3482.

[246] O. Hara, H. Anzai, S. Imai, Y. Kumada, T. Murakami, R. Itoh, E. Takano, A. Satoh, and K. Nagaoka, *J. Antibiot.*, 1988, **41**, 538.

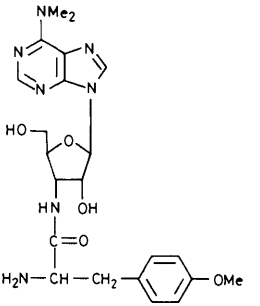


Erythromycin-A $R^1 = OH$ $R^2 = Me$
-B $R^1 = H$ $R^2 = Me$
-C $R^1 = OH$ $R^2 = H$
-D $R^1 = R^2 = H$

(103)



oleandomycin
(104)



puromycin
(105)

localized, although recent results[246] have identified a set of multiple genes controlling the alanylation steps. The cloned resistance gene has been expressed[247] in *E. coli* and *S. lividans*.

The erythromycins (103) are clinically important antibiotics, produced by *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*[248]). The erythromycin resistance gene has been cloned,[249] using the techniques described above, and used as a hybridization probe to screen a library of *S. erythraea* DNA fragments in a bifunctional *Streptomyces/E. coli* cosmid. One clone (pKC488) was selected from this library on the basis of its ability to hybridize to the probe, and this contained an insert of about 35 kb which included not only the resistance gene but also some of the erythromycin biosynthetic genes.[250] Thus the cosmid was able to complement mutants blocked in erythromycin biosynthesis, and although the vector was unstable, when introduced into *S. lividans*, a strain which does not normally produce macrolides, t.l.c. evidence was obtained for the production of low levels of erythromycin. This remarkable result suggests that the 35 kb insert in this cosmid may contain a complete set of genes for

[247] E. Strauch, *et al.*, *Gene*, 1988, **63**, 65.
[248] D. P. Labeda, *Int. J. System. Bacteriol.*, 1987, **37**, 19.
[249] C. J. Thompson, T. Kieser, J. M. Ward, and D. A. Hopwood, *Gene*, 1982, **20**, 51.
[250] R. Stanzak, P. Matsushima, R. H. Baltz, and R. N. Rao, *Biotechnology*, 1986, **4**, 229.

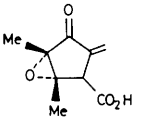
erythromycin production, thus allowing its formation in *S. lividans*, or that it possesses a partial set of genes that complement a partial set in the host. More evidence about the functional role of this cloned DNA is eagerly awaited.

In related work, a genomic library of DNA fragments from the oleandomycin (104) producer *S. antibioticus*, in the plasmid pNJ1 (which carries a gene for thiostreptone resistance), was used to transform a mutant of *S. erythraea* blocked in erythromycin production at a stage prior to macrolide ring formation.[178] One out of 288 thiostreptone-resistant clones generated in this way had regained the ability to produce antibiotic activity. The antibiotic substances were isolated and shown to have the structural formulae shown in (80), corresponding to 2-norerythromycins-A, -B, -C, and -D. It is noteworthy that neither oleandomycin nor the erythromycins have a macrolide ring lacking the 2-methyl substituent. It is possible that the inactive PKS in the mutant strain of *S. erythraea* has been repaired and become functional again by gaining a protein encoded by the plasmid borne sequences from *S. antibioticus*. This fascinating observation at least provides an indication that the activities of the macrolide PKSs may be engineered for the production of novel macrolide frameworks.

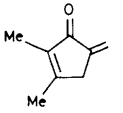
The final example in this section is puromycin (105), an aminonucleoside antibiotic which inhibits protein biosynthesis by interacting with the A-site of the large ribosomal sub-unit; puromycin is the amide analogue of the terminal unit of tyrosinyl-tRNA, is taken up by the ribosome in place of the latter, and blocks the subsequent formation of a new peptide bond.[251] Cell-free extracts of *S. alboniger* contain a puromycin *N*-acetyl transferase, which acetylates the free amino group and affords the inactive *N*-acetylpuromycin.[252] The resistance gene encoding this protein has been cloned,[253] using pIJ702 as vector and *S. lividans* as host. This time, clones in which the *S. alboniger* DNA inserts in the vector extended to one side of the resistance gene gave rise to enzymic activity, in cell-free extracts, for the final step in puromycin biosynthesis, an *O*-methyl-transferase.

D. Use of Synthetic Oligonucleotide Probes.—Applications of this method have been described already, and include those in the β-lactam field, and in the biosynthesis of the macrolide tylosin. Thus in the latter case,[184] N-terminal sequence information on a macrocin-*O*-methyltransferase purified from *S. fradiae*, was used to synthesize a 44-base mixed oligonucleotide probe, which could be used to screen a lambda library in *E. coli*. Recombinants that contained overlapping fragments of chromosomal DNA were isolated, and these included sequences that encode the methyltransferase. Analysis of the flanking DNA revealed genes for several other steps in the tylosin pathway, and included a tylosin resistance gene. However, no DNA sequences were found that could complement mutants blocked before the formation of tylactone (70), which should include the tylactone PKS. It appears that genes for tylosin biosynthesis

[251] E. Cundliffe, *Br. Med. Bull.*, 1984, **40**, 61.
[252] J. Vara, J. A. Perez-Gonzalez, and A. Jimenez, *Biochemistry*, 1985, **24**, 8074.
[253] J. Vara, F. Malpartida, D. A. Hopwood, and A. Jimenez, *Gene*, 1985, **33**, 197.



methylenomycin-A
(106)



methylenomycin-B
(107)

may be located in more than one region of the *S. fradiae* genome. An *O*-methyltransferase acting in the avermectin pathway has also been described.[254]

E. Mutational Cloning.—The mutational cloning strategy developed by Chater and co-workers (described in references 255 and 256) makes use of a Streptomycete phage vector, and has been used to isolate genes for the production of methyleneomycins A and B [(106) and (107)]. The methylenomycins are unusual in that the biosynthetic and resistance genes are located upon a plasmid,[257,258] SCP1, in *S. coelicolor*. The plasmid has been difficult to purify as an intact molecule due to its large size (around 500 kb). Recently evidence for the involvement of giant linear plasmids in the biosynthesis of other antibiotics in Streptomycetes has been described.[259] Analysis of the cloned [255,260] methylenomycin biosynthetic genes revealed that the region consists of at least 17 kb of SCP1 DNA, and includes a resistance gene, at least four structural genes, and a regulatory gene. Few details are currently known about the pathway,[261] except that the last step involves an epoxidation of a cyclopentenone intermediate.

F. Exploiting Homologies between PKS Genes.—The actinomycetes are known to produce many hundreds of different polyketide antibiotics, and it seems reasonable to speculate that the genes coding for the several hundred different PKS complexes needed to synthesize these products should not have evolved independently. Given this, it is possible that the divergence of *pks* genes from a common ancestor would have left sufficient sequence homologies between them to allow the use of one such *pks* gene to detect and isolate others, by Southern hybridization analysis. This idea was tested by Hopwood and co-workers[262] using the *act* I gene, which is believed to encode the condensing enzyme of the actinorhodin PKS, as a hybridization probe. Discrete bands of varying intensity

[254] M. D. Schulman, D. Valentino, M. Nallin, and L. Kaplan, *Antimicrob. Agents Chemother.*, 1986, **29**, 620.
[255] K. Chater and C. J. Bruton, *Gene*, 1983, **26**, 67.
[256] M. R. Rodicio, C. J. Bruton, and K. F. Chater, *Gene*, 1985, **34**, 283.
[257] R. Kirby, L. F. Wright, and D. A. Hopwood, *Nature*, 1975, **254**, 265.
[258] L. F. Wright and D. A. Hopwood, *J. Gen. Microbiol.*, 1976, **95**, 96.
[259] H. Kinashi, M. Shimaji, and A. Sakai, *Nature*, 1987, **328**, 454.
[260] K. F. Chater and C. J. Bruton, *EMBO J.*, 1985, **4**, 1893.
[261] Reviewed by U. Hornemann and D. A. Hopwood, in 'Antibiotics Vol. IV, Biosynthesis', ed. J. W. Corcoran, Springer-Verlag, New York, 1981.
[262] F. Malpartida, S. E. Hallam, H. M. Kieser, H. Motamedi, C. R. Hutchinson, M. J. Butler, D. A. Sudgen, M. Warren, C. McKillop, C. R. Bailey, G. O. Humphreys, and D. A. Hopwood, *Nature*, 1987, **325**, 818.

hybridizing to *act* I were found in restriction digests of genomic DNA from *S. coelicolor* (as control) and 14 out of 17 other Streptomycetes known to produce polyketides, including those making anthracyclines (tetracenomycin, adriamycin), macrolides (spiramycin, tylosin, pikromycin, oleandomycin), polyethers (salinomycin, lasalocid, monensin), macrotetrolides (nonactin), milbemycins, as well as others of the isochromanequinone class (granaticin, medermycin, and kalafungin). Of the others tested that are known polyketide producers, only strains producing oxytetracycline, erythromycin, candicidin, and curamycin showed no hybridizing band. Of the seven strains examined that were not known to produce polyketides, only two, a chloramphenicol-producing *S. venezuelae* and an actinomycin-producing *S. parvulus* showed hybridizing bands. However, these two strains have been shown subsequently to have a cryptic capacity for polyketide production.[262] The *act* III probe also revealed hybridizing bands in most of the same strains as the *act* I probe, but significantly not in the producer of tetracenomycin whose biosynthesis in any case would not require a reduction during chain assembly by the PKS (Scheme 26).

These results demonstrate a correlation between polyketide production and *act* I/*act* III homologous sequences, but do not constitute proof that these sequences indeed represent the respective PKS genes present in these strains. Nevertheless, several such proofs have been obtained. In the case of the tetracenomycin gene cluster, isolated independently (*vide supra*), a region of about 2 kb was shown to hybridize to *act* I, explaining the appearance of a hybridizing band of this size in genomic digests of *S. glaucescens*. Similar experiments identified regions with weak homology to *act* I and *act* III in cloned *S. rimosus* DNA carrying genes for oxytetracycline production. Homology between the *act* probes and the *otc* DNA was much weaker than that seen with the *tcm* DNA, correlating with the failure to observe hybridizing bands in genomic digests of *S. rimosus*, mentioned earlier. One other test of the hypothesis involved *S. violaceoruber*, the producer of granaticin. Here overlapping segments of genomic DNA from *S. violaceoruber* were cloned in the lambda EMBL4 vector, by selection with *act* I and *act* III. This DNA, and adjacent fragments of the clones, were found to complement *S. coelicolor act* mutants of classes I, II, III, and IV, and to give rise to blocked mutants in gene disruption experiments with an *att*-site deleted ΦC31 phage vector, demonstrating its functional role in granaticin biosynthesis. Similar results[262] were obtained for the corresponding fragments cloned from the milbemycin producer, *S. hygroscopicus* ssp. *aureolacrimosus*.

These very encouraging results support the view that these probes may be of value in the isolation of genes for related polyketide synthase complexes. This should open the way to a more detailed knowledge of the primary structures of these proteins, and of the structural relationships that exist between them.

[263] T. Ohnuki, *et al.*, *J. Bacteriol.*, 1986, **164**, 85.
[264] T. Schupp, C. Toupet, and M. Divers, *Gene*, 1988, **64**, 179.
[265] C. W. Chen, H.-F. Lin, C. L. Kuo, H.-L. Tsai, and J. F.-Y. Tsai, *Biotechnology*, 1988, **6**, 1222.