N. J. HARPER and A. B. SIMMONDS

Advances in DRUG RESEARCH

Volume 1

Advances in

DRUG RESEARCH

Edited by

N. J. HARPER

Allen & Hanburys Ltd
Ware, Hertfordshire, England

AND

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London, England

VOLUME 1

1964

ACADEMIC PRESS · LONDON AND NEW YORK

ACADEMIC PRESS INC. (LONDON) LTD
BERKELEY SQUARE HOUSE
BERKELEY SQUARE
LONDON. W.1

U.S. Edition published by

ACADEMIC PRESS INC
111 FIFTH AVENUE
NEW YORK, NEW YORK 10003

Library of Congress Catalog Card Number: 64–24672

PRINTED IN GREAT BRITAIN BY
SPOTTISWOODE, BALLANTYNE & CO. LTD
LONDON AND COLCHESTER

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Preface

Drug research involves the interplay of various scientific disciplines each with its specialized literature. The problem of keeping reasonably well informed, even in one of the many areas of drug research is well recognized.

"Advances in Drug Research" provides a medium for the presentation of significant current progress to chemists, biochemists and pharmacologists who are interested in the chemical and biological aspects of drugs. The series is designed to provide comprehensive surveys, not only in established areas of drug research but also in the newer and sometimes relatively narrower, more exploratory areas. The reviews will collate existing knowledge and it is hoped will point the way to valuable general and new hypotheses. Authors will be encouraged to speculate, since speculation is a stimulus to directed experimentation, which in turn leads to increased understanding.

In Volume I there is a comprehensive review of Penicillins and Related Structures by workers well known for their original contributions in this field. A survey of Antitussives, a topic hitherto inadequately documented, should be of interest to many. Physicochemical aspects of drug action rightly claim increasing attention and a well known American authority writes on the Physiological Transport of Drugs. Interest in anti-hypertensive drugs is widespread and a review of Adrenergic Neurone Blocking Agents covers one aspect of this field of research.

N. J. HARPER

June 1964 A. B. SIMMONDS

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Penicillins and Related Structures

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1. Products of Simple Fermentation

1.1. Early work

The historic discovery by Sir Alexander Fleming of a powerful antibacterial agent produced by a mould, subsequently identified as a strain of *Penicillium notatum*, has been described too often to require elaboration. Fleming (1929) named the active substance penicillin, but with the limited resources and techniques then available he was unable to effect its isolation. The acidic nature of penicillin was indicated by the work of Raistrick and his colleagues (Clutterbuck *et al.*, 1932), who found that very little of the antibiotic activity was extracted into ether from a neutral aqueous solution, whereas at pH 2 extraction was almost complete. In the course of these early investigations it was observed that solutions of penicillin were moderately stable at pH 5 to 6 and normal temperature, but very labile towards acid, alkali, oxidants and heat.

The instability of penicillin continued to frustrate isolation attempts until the problem was taken up by Chain and Florey at Oxford. By applying both extraction and chromatographic procedures the Oxford team prepared a penicillin salt in solid, though impure, form and demonstrated its effectiveness against various pathogens in experimental animals (Chain *et al.*, 1940) and, shortly afterwards, in man (Abraham *et al.*, 1941). Recognition of the potential value of penicillin resulted in the inauguration of a vast co-operative research effort in Britain and the United States of America. The results of this work were not published at the time, but an excellent general account was given after the war by Florey and his colleagues (1949), whilst a truly massive monograph has been devoted to the chemical aspects alone (Clarke *et al.*, 1949).

The rapid development of the fermentation process for the production of penicillin from a laboratory operation to a major industry owes much to two advances initiated at the Northern Regional Laboratories, Peoria, Illinois (Moyer and Coghill, 1946). One was the replacement of surface culture of the mould by deep fermentation, thus permitting the use of large capacity fermenters, and the second was the selection of high-yielding mould strains. The strains of *Penicillium chrysogenum* now used industrially give titres many times greater than the original *Penicillium notatum*.

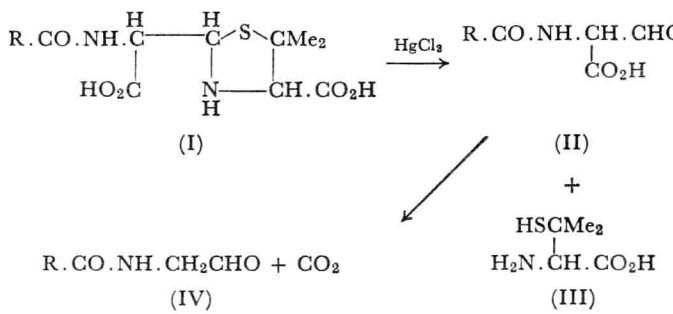
1.2. Elucidation of structure

It would be beyond the scope of the present account to review all the evidence bearing on the structure of penicillin, for which the reader is referred to "The Chemistry of Penicillin" (Clarke *et al.*, 1949) and to

summaries by Cook (1948) and Robinson (1950). Whilst the final solution of the problem resulted from the collaboration of many groups it should not be overlooked that the greatest contribution, particularly in the earliest and most difficult phase, was made by the Oxford team of Abraham, Baker, Chain, and Robinson. In the resumé which follows the facts are arranged in the order considered most suited to clarity rather than that in which the various discoveries were made.

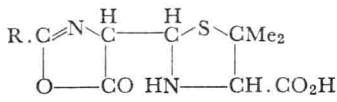
As soon as crystalline specimens of penicillin salts became available it was evident that the material prepared in America was not identical with the British product. It is now recognized that, depending on the strain of mould used and the composition of the fermentation medium, various penicillins differing with respect to a single acyl radical may be formed. Vigorous hydrolysis liberates this group in the form of a carboxylic acid, $R.CO_2H$, which is characteristic of the particular penicillin. The earliest American penicillin gave phenylacetic acid in this way, whilst its British counterpart afforded hexenoic acid. In naming individual penicillins the name of the radical (R), generally known as the side chain, is employed as a prefix. The remainder of the molecule, called the nucleus, is the same in all penicillins. It represents the labile portion of the molecule and hence is disrupted during the hydrolytic removal of the side chain. Elucidation of the structure of the nucleus involved extensive degradative studies of which only the more significant are mentioned here.

Mild hydrolysis of a penicillin involves the addition of the elements of one molecule of water to give the appropriate dibasic penicilloic acid (I). The structure of the latter was deduced by degradation with mercuric chloride to give D-penicillamine (III), the penilloaldehyde (IV), and carbon dioxide. Various penicilloic acid derivatives were also synthesized from penicillamine and derivatives of the appropriate penaldic acid (II).



Penicillin itself, a monocarboxylic acid without basic properties, was obviously a dehydration product of penicilloic acid (I) and the choice

evidently lay between the oxazolone structure (V) and the β-lactam (VI). On general grounds most chemists at first favoured the oxazolone formula (V), but later the fused ring system (VI) came to be preferred (Robinson, 1950). Chemical evidence for the latter came from desulphurization studies in the Merck laboratories (Kaczka and Folkers, 1949). One of the products obtained by treating a hot aqueous solution of benzylpenicillin with a fairly active form of Raney nickel proved to be dethiobenzylpenicillin (VII), in which the β-lactam ring was preserved intact. Elegant and conclusive proof of the β-lactam structure was finally provided by X-ray crystallographic analysis of penicillin salts (Crowfoot *et al.*, 1949). Since structure (VI) contains three asymmetric carbon atoms, penicillin actually represents only one of eight possible stereochemical forms.



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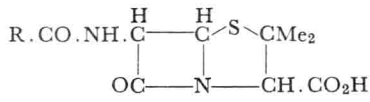

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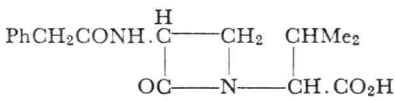

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1.3. THE "NATURAL" PENICILLINS

Table I lists the penicillins which have been definitely identified as major products of unaided fermentation. The first five have been known since 1945 and have all been isolated in essentially pure form and their structures established by the degradative procedures outlined above. Since that time, however, detection of the various penicillins in a fermentation brew has been greatly facilitated by the development of paper chromatography. Goodall and Levi (1947) applied this technique to penicillin mixtures by allowing them to run in a suitable solvent system on a paper strip in the usual way, and then transferring the strip to an agar plate seeded with a penicillin-sensitive organism. After incubation, zones of inhibition of bacterial growth were revealed in the agar corresponding to the positions of the various penicillins on the strip. In this way Goodall and Levi demonstrated the presence in fermentation broths of small quantities of several unidentified penicillins in addition to the known ones. Several other workers (Winsten and Spark, 1947; Karnovsky and Johnson, 1949; Hale *et al.*, 1953) have made similar observations.

The sixth penicillin listed in Table I was characterized by Newton and Abraham (1954) and is unusual in containing a side-chain derived from D-α-aminoadipic acid. It is formed by certain *Cephalosporia*, such as *Cephalosporium salmosynnematum* and hence was originally known in Britain as Cephalosporin N and in the United States as Synnematin B (Gottshall *et al.*, 1951; Abraham *et al.*, 1955). The name Cephalosporin N is still widely used but is undesirable because it may lead to confusion with antibiotics of the Cephalosporin C group, containing a different nucleus, which will be discussed later. For this reason the present authors have adopted the suggestion of Newton and Abraham (1955) that (D-4-amino-4-carboxybutyl)penicillin be called Penicillin N. The polar and hydrophilic nature of the side chain necessitates the use of different isolation procedures for Penicillin N than those used for the other penicillins.

TABLE I

The "Natural" Penicillins

Common name	*Chemical name*	*Side-chain (R) in formula (VI)*
Penicillin F	Pentenylpenicillin	$CH_3CH_2CH:CHCH_2$
Penicillin dihydro F	Pentylpenicillin	$CH_3(CH_2)_4$
Penicillin K	Heptylpenicillin	$CH_3(CH_2)_6$
Penicillin G	Benzylpenicillin	$PhCH_2$
Penicillin X	*p*-Hydroxybenzylpenicillin	$HO.C_6H_4.CH_2$
Penicillin N	(D-4-Amino-4-carboxybutyl)penicillin	$H_2N.CH(CO_2H).(CH_2)_3$

Recent observations (Flynn *et al.*, 1962) indicate that *Penicillium chrysogenum* probably produces small amounts of the epimer of Penicillin N, having a side chain derived from L(+)α-aminoadipic acid.

2. BIOSYNTHESIS

Elaboration of the penicillin molecule by suitable moulds takes place within the cells, and the end product is then excreted into the culture medium, less than 1% being retained inside the mycelium (Demain, 1957). Since biosynthesis of penicillin proceeds even when the mould is grown on a synthetic nutrient medium the process must start from very simple molecules and involve many steps. The earlier stages are undoubtedly closely associated with general cell metabolism and will not be considered here, but the later ones, involving the incorporation

of more complex "precursors" into the antibiotic molecule, merit discussion.

The first steps in elucidating the biosynthetic mechanism were taken during attempts to improve penicillin titres, and throughout the development of the subject academic and practical considerations have remained inextricably mixed. An obvious useful result has been the development of a wider range of penicillins than those previously known.

2.1. ORIGIN OF THE SIDE CHAIN

The early specimens of penicillin produced in this country consisted largely of the aliphatic Penicillin F and Penicillin K, whereas the major constituent of the American product was Penicillin G. The yield of benzylpenicillin in the American process was increased by the use of corn steep liquor in the fermentation medium. This nutrient material contains benzenoid substances such as phenylalanine and β-phenylethylamine which appeared to be incorporated into the side chain of the penicillin. Various investigators therefore tried the effect of adding these and other substances containing the benzyl group to the fermentation medium. Introduction of phenylacetic acid (Moyer and Coghill, 1947) and several of its derivatives, such as the simple and *N*-substituted amides, was found to stimulate production of benzylpenicillin and so, curiously enough, did the addition of corresponding derivatives of γ-phenylbutyric acid (Behrens, 1949). The possibility that such substances were merely acting as non-specific growth promoters was eliminated by examination of the benzylpenicillin isolated following the use of deuterophenylacetyl-N^{15}-valine as a precursor. Deuterium analyses demonstrated that the phenylacetyl portion of the precursor was incorporated directly into the penicillin, whereas very little labelled nitrogen was found in the product (Behrens *et al.*, 1948b).

When phenylacetic acid derivatives are used in adequate amounts as side-chain precursors benzylpenicillin is produced in good yield, to the virtual exclusion of the other "natural" penicillins. The formation of a single penicillin, together with the improved yield, greatly simplify isolation of the antibiotic. Chromatographic techniques are no longer necessary, the product being simply extracted into an organic solvent at low pH, and then converted directly into a salt by treatment with a suitable inorganic or organic base. Such is the basis of the modern production of Penicillin G, the only one of the "natural" penicillins now produced commercially.

Formation of *p*-hydroxybenzylpenicillin (Penicillin X) can also be stimulated by the addition of *p*-hydroxyphenylacetic derivatives, but no efficient precursors have been found for the other penicillins of Table I.

Derivatives of straight chain aliphatic acids are incorporated into the penicillin side chain to some extent but more than one penicillin usually results, apparently due in part to stepwise breakdown of the precursor by β-oxidation. Thus the addition of octoic acid, conveniently in the form of its glyceryl ester, results in only a very slight increase in the production of heptylpenicillin together with a somewhat greater increase in that of pentylpenicillin. Production of several of the unidentified "natural" penicillins referred to previously appears to be stimulated by certain aliphatic precursors, suggesting that some of these minor products are lower alkyl penicillins (Thorn and Johnson, 1950).

The successful use of side-chain precursors is not, however, restricted to the production of "natural" penicillins. Thus many ring-substituted phenylacetic acid derivatives have proved acceptable to the mould and several of the resulting substituted benzylpenicillins have been isolated in pure form (Behrens *et al.*, 1948a). Several substituted thienylmethylpenicillins have similarly been produced with the aid of suitable precursors. Another type of side chain which lends itself to incorporation by the precursor technique is that containing an oxygen or sulphur atom in the β-position. Thus derivatives of phenoxyacetic acid and 2-phenoxyethanol serve as efficient precursors for phenoxymethylpenicillin (Penicillin V), whilst allylthiomethylpenicillin (Penicillin O) and butylthiomethylpenicillin (Penicillin S) result from the use of appropriate alkylthio-acetic acid derivatives (Behrens *et al.*, 1948a; Brandl and Margreiter, 1954).

In view of the very inefficient incorporation of straight-chain monocarboxylic acids into the penicillin side chain, the successful use of α,ω-dicarboxylic acids as precursors (Ballio *et al.*, 1960) is rather unexpected. Using adipic acid as a precursor, these workers were able to prepare 4-carboxybutylpenicillin in moderately pure form.

By no means all carboxylic acids or their derivatives, even when nontoxic to the mould, can function as penicillin side-chain precursors. Indeed, all the precursors which have been definitely proved suitable contain the terminal group CH_2CO_2H or its equivalent. Attempts to incorporate di- or tri-substituted acetic acids, or compounds in which the carboxyl group is directly attached to a cyclic structure, have invariably failed (Mortimer and Johnson, 1952). This limitation presumably reflects the specificity of one or more of the enzymes involved in the biosynthetic process.

When a readily accepted precursor is supplied in adequate amount the corresponding biosynthetic penicillin may be produced to the virtual exclusion of the "natural" penicillins. With precursors that are less well accepted, however, the new penicillin usually represents only a part of

the total antibiotic yield. Furthermore, an increase in total antibacterial activity is not necessarily observed, particularly if the new penicillin has relatively poor activity against the test organism in use. For these reasons by far the best method of determining whether or not a given side-chain precursor is accepted by the mould is by paper chromatography. Although earlier studies (Behrens *et al.*, 1948a; Behrens, 1949) in which this technique was not used led to the isolation of a number of new penicillins, they also included many inconclusive experiments.

2.2. ORIGIN OF THE NUCLEUS

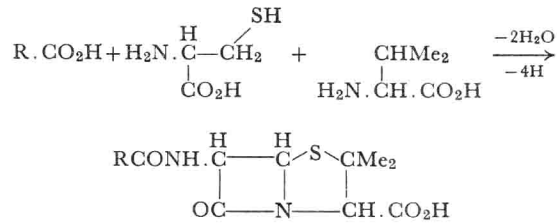
The precursors of the penicillin nucleus proved less easy to identify than those of the side chain, because they do not usually stimulate the antibiotic yield. This is presumably because the appropriate biosynthetic steps are not rate-limiting. Progress in this direction has therefore stemmed largely from a long series of immensely thorough radioactive tracer studies.

Stevens and co-workers (1953, 1954a) studied the possible sulphur-containing precursors by comparing the uptake of sodium [^{35}S] sulphate into penicillin in their presence and absence. Although the typical sulphur-containing degradation product of penicillin is penicillamine, this amino-acid does not appear to be a biosynthetic precursor since it does not compete effectively with labelled sulphate for incorporation into the antibiotic. Compounds which do compete effectively with sulphate as sources of the penicillin sulphur atom include homocysteine, methionine and cystathionine, but more effective than any of these is L-cystine. This result was confirmed in a similar experiment with L[^{35}S] cystine and unlabelled sulphate. By contrast, D-cystine does not compete successfully with labelled sulphate.

Utilization of L-cystine in penicillin biosynthesis is not, however, confined to the sulphur atom since equal incorporation of isotopic carbon, nitrogen and sulphur from L-[β- ^{14}C : ^{15}N : ^{35}S] cystine has been demonstrated (Arnstein and Grant, 1954). Cystine is probably first reduced to cysteine, the skeleton of which is incorporated intact with retention of the L-configuration. Such findings prove conclusively that the central portion of the penicillin molecule, comprising the sulphur atom, the three carbon atoms of the β-lactam ring, and the exocyclic nitrogen atom, is derived from cystine.

Having demonstrated the incorporation of the side-chain acid and of cysteine into penicillin it is easy to see, as Scheme 1 shows, that the remainder of the molecule is probably derived from valine or a closely related compound. Studies with ^{14}C-labelled valine have, in fact, demonstrated the incorporation of the complete carbon skeleton of this

amino-acid into penicillin (Stevens *et al.*, 1954b; Arnstein and Grant, 1954; Arnstein and Clubb, 1957). On degradation the radioactivity is found almost exclusively in the penicillamine fragment. Other compounds containing the same carbon skeleton, such as β-hydroxyvaline, ββ-dimethylacrylic acid, αβ-dihydroxyisovaleric acid and α-ketoisovaleric acid, are not utilized as efficiently as valine (Stevens and DeLong, 1958).



SCHEME 1. Representation of the principal biosynthetic precursors of penicillin.

The extent to which the nitrogen atom of valine is incorporated into penicillin varies with the experimental conditions. Arnstein and Clubb (1957) observed much greater dilution of labelled nitrogen than of labelled carbon, but this could be due to participation of valine in transamination reactions prior to incorporation in the antibiotic. In short-term experiments isotopic valine nitrogen was utilized relatively efficiently (Stevens and DeLong, 1958).

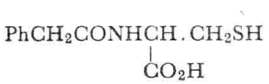
The most curious aspect of the utilization of valine is that, despite the fact that the penicillamine component of penicillin has the D-configuration, this fragment is assimilated more readily from L-valine than from the D-enantiomorph (Stevens *et al.*, 1956; Arnstein and Clubb, 1957; Stevens and DeLong, 1958). Evidently inversion occurs at some stage in the biosynthesis, possibly via an αβ-unsaturated intermediate.

In contrast to its ability to incorporate many biologically foreign acids into the penicillin side chain, the mould appears to be unable to synthesize analogues with modified nuclei. The provision of amino-acids other than cystine and valine either fails to affect or actually inhibits biosynthesis (Arnstein and Margreiter, 1958).

2.3. MECHANISM

Identification of the three principal biosynthetic precursors accounts for all the atoms in the penicillin molecule (Scheme 1) but tells us little about the order and manner in which these components combine. The dehydrogenation steps are particularly difficult to elucidate, although some of the possibilities have been eliminated. For example, studies with tritium-labelled cystine have shown that the α-hydrogen atom and one

of the two β-hydrogen atoms are retained (Arnstein and Crawhall, 1957). Neither *N*-phenylacetylcysteine (VIII) nor $\beta\beta$-dimethyllanthionine (IX) appears to be incorporated intact into the benzylpenicillin molecule. The disulphide of L-cysteinyl-L-valine (X) appears to be directly incorporated into penicillin to a limited extent only, suggesting that an alternative pathway also exists (Arnstein and Morris, 1960). It is, of course, possible that in the course of biosynthesis one or more of the components may combine with other molecules (not shown in Scheme 1) which are subsequently eliminated.



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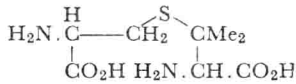

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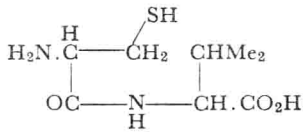

(X)

At this stage it is appropriate to consider other approaches to the problem of penicillin biosynthesis. Although some of these lack the rigour of the tracer experiments they have yielded results no less interesting. Hockenhull (1949) observed that when the mycelium of *Penicillium notatum* growing in a synthetic medium containing no added side-chain precursor was transferred to a medium containing phenylacetic acid, enhanced penicillin production commenced forthwith. Conversely, mycelium grown in a medium containing phenylacetic acid ceased to produce appreciable quantities of benzylpenicillin when transferred to a medium which did not contain the acid. Hockenhull deduced that the mould produced some precursor which was capable of direct reaction with phenylacetic acid in the medium to yield benzylpenicillin, and that the side chain was not first stored by the mould. This would suggest that incorporation of the side chain is either the last stage (Hockenhull *et al.*, 1949), or at any rate the last rate-limiting stage, in penicillin biosynthesis.

Burger (1951) made a similar suggestion, apparently on more intuitive grounds. He considered that the ability of the mould to produce a variety of penicillins pointed to a final acylation of a common intermediate (XI). The hypothetical structure (XI) had been recognized much earlier as being, in a purely formal sense, the parent amine of the penicillin series. Kühne (1946) had indeed suggested that it be assigned the trivial name "Penine" and that the various penicillins be re-named as acylpenines.