ASSOCIATION OF 6-OXO-PIPERIDINE-2-CARBOXYLIC ACID WITH PENICILLIN V PRODUCTION IN *PENICILLIUM CHRYSOGENUM* FERMENTATIONS

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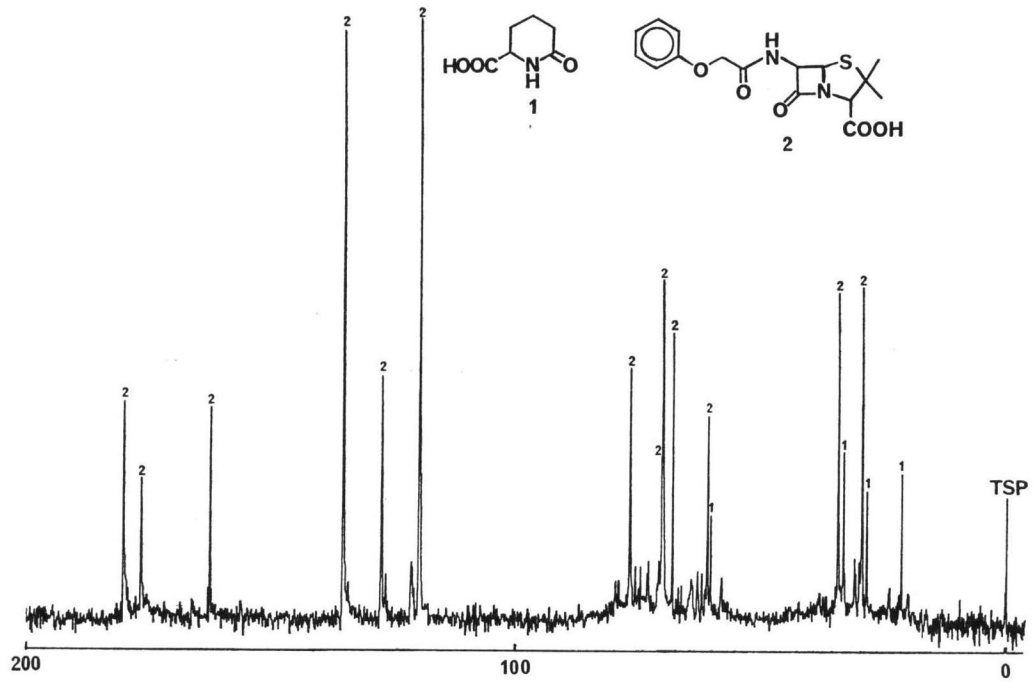
Analysis of a ^{13}C NMR spectrum of a concentrated broth from *Penicillium chrysogenum* fermentation revealed the presence of penicillin V and 6-oxo-piperidine-2-carboxylic acid (**1**) as the principal constituents. The latter lactam, identical to an authentic sample prepared by the cyclization of α-aminoadipic acid was present to the extent of 28 mol% of penicillin V. The lactam isolated from the broth was nearly racemic, having a slight excess of the L-isomer. This isolation provides further evidence regarding the biosynthetic precursors of the hydrophobic penicillins.

As part of our continuing studies of the production of antibiotics, we are examining several commercial fermentations by various NMR techniques. NMR procedures are routinely used to characterize fermentation products. However, few applications as a means to monitor fermentation behavior have been reported. In contrast to proton NMR spectra which typically consist of broad overlapping peaks, we have found that ^{13}C spectra of fermentation broths without any purification provide useful insights. ^{13}C spectra are useful because of the plethora of well-resolved peaks in the usual proton-decoupled spectra. The major limitation of the procedure is the sensitivity of the spectrometer.

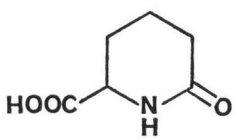
An examination of the ^{13}C spectrum (Fig. 1) of a typical *Penicillium chrysogenum* penicillin V broth concentrate quickly reveals the extent of penicillin production in the utilization of media constituents. The preponderance of penicillin V over other organic materials can be discerned as well as the presence of a second major component. The upfield positions of the carbon signals of the unknown were consistent with a substance related to penicillin. However, off-resonance proton decoupling experiments established that the three new, higher field peaks were associated with carbons having two attached hydrogens. This fact immediately led to the suggestion that the unknown compound was 6-oxo-piperidine-2-carboxylic acid, the lactam produced by the cyclization of α-aminoadipic acid. The peaks of a ^{13}C spectrum of an authentic sample of the lactam, prepared by the procedure given by GREENSTEIN and WINITZ[1] were coincident with those arising from the suspected second component in the broth.

This assignment was confirmed by isolation of the new substance and direct comparison. A sample from a filtered lyophilized broth was triturated with methanol. The filtered extract was concentrated to dryness and the residue was dissolved in water. After adjustment of the pH to 2.0 with dilute HCl, the solution was again reduced to dryness *in vacuo*. The resulting solid was chromatographed on a silica column with acetone, toluene, acetic acid and water (50: 15: 10: 15, v/v) as eluant. Material from appropriate fractions was recrystallized from acetone or methanol. This was identical to lactam 1 prepared from D-α-aminoadipic acid by IR, PMR, CMR, MS, and TLC.

Fig. 1. 20 MHz CMR spectrum of *Penicillium chrysogenum* broth lyophilized and reconstituted at pH 7.2; 57,000 transients, 48° Tip angle, at 1.023 sec., SW 4,000 Hz (200 ppm).



The amount of the piperidone-carboxylic acid in the broth was quantitated by an application of a chemical assay procedure for penicillin.[2] In this the lactam was converted to a *p*-bromophenacyl ester which was analyzed by HPLC using a Waters C-18 μ Bondapak reverse phase system (65% methanol - H_2O). Quantitation was performed by adding known amounts of authentic lactam and penicillin V in separate experiments to lyophilized broths after reconstitution, followed by derivatization and HPLC. Penicillin V was also assayed by an alternate HPLC procedure that did not involve formation of a derivative. The lactam was present to the extent of 28 mol% of the concentration of penicillin V. The estimation by ^{13}C NMR was somewhat higher than this value. Interestingly, the amount of lactam compared to the level of penicillin was relatively invariant with respect to the stage of the fermentation cycle as judged by NMR experiments. The production of the lactam in substantial quantity in the commercial fermentation has obviously been undetected due to its lack of antibacterial activity, a ninhydrin response, and a UV chromophore.



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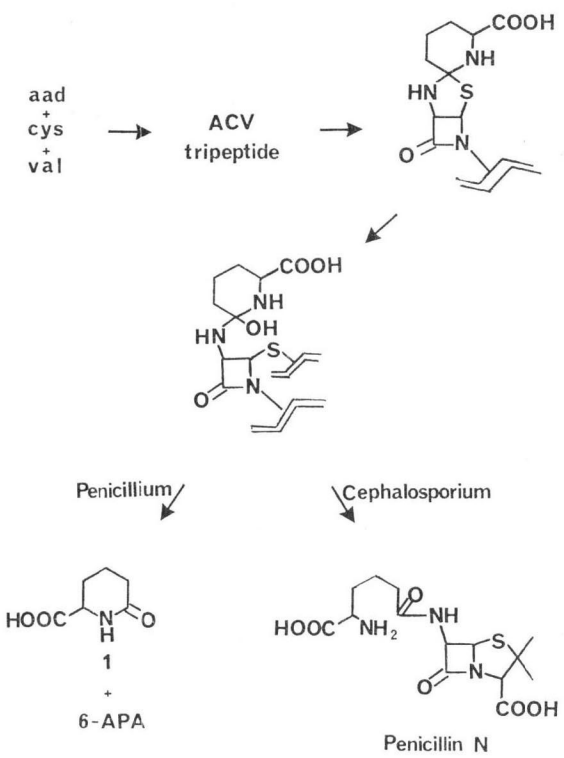
The lactam was also found in significant quantities in penicillin G fermentation precursored with phenylacetic acid. The lactam was present in both large tank and shake flask fermentations. In a non-precursored *Penicillium* fermentation the lactam was formed in nearly equal proportions to 8-hydroxypenillic acid, derived from the CO_2 adduct of 6-APA.[3] The lactam was not found in zero time autoclaved fermentation media. Further it was not detected in broths from non-antibiotic producing strains of *Penicillium* or in broths from *Cephalosporium* fermentations producing various cephalosporin

antibiotics*. No significant amount of α-aminoadipic acid could be found in the *Penicillium* fermentations by ^{13}C NMR or by TLC using a ninhydrin spray.

Clearly, the formation of the lactam acid is closely associated with production of 6-APA and derived biosynthetic penicillins. The formation of 6-APA and the lactam in the final biosynthetic stages in *Penicillium* and penicillin N in *Cephalosporium* has been hypothesized earlier to result from alternate cleavages of orthoamide structures which could be important in the biosynthesis of these antibiotics.[5] Efforts to gain supportive chemical evidence for orthoamide involvement were thwarted. The group at Oxford[6,7] has reported that several broken-cell preparations from *P. chrysogenum* will catalyse the conversion of isopenicillin N or 6-APA to penicillin G in the presence of phenylacetylcoenzyme A. Our results would suggest that if this is the major pathway in the intact cell the enzymatic product is the piperidine-2-carboxylic acid and not α-aminoadipic acid. Presumably, the amino group of the aminoadipic acid participates in facilitation of the acylase transfer. Similarly, pyrolidone-2-carboxylic acid is involved in the γ-glutamyl cycle.[8]

Scheme 1.



The Oxford work indicates that isopenicillin N and not penicillin N is the substrate for their described acyltransferase system. This suggests that our isolated lactam should have the L-configuration. However, we find that the piperidone carboxylic acid from the *P. chrysogenum* broth is essentially racemic with a slight excess of the L-isomer. In one isolated sample the rotation was $[\alpha]_D$ 4.4, *c* 0.225, MeOH compared to $-24.8°$, *c* 0.25, MeOH, (-15.5, 2% in H_2O; -41.3, 2% in 6 N HCl) for the authentic D-isomer.[4] In a separate isolation from a different broth, following somewhat modified conditions, the specific rotation was $[\alpha]_D$ 5.8°**. FRIEDRICH and DEMAIN[9] have concluded from their studies on resting cells of *P. chrysogenum* that α-aminoadipic acid is extensively recycled and several molecules of penicillin (10 in one example) can arise per molecule of α-aminoadipic acid. In their specific system, using 1-^{14}C-DL-α-aminoadipic acid, they found α-keto adipic acid was the only metabolite which was formed to the extent of 25% of the added amino acid. These results indicate further studies are needed to understand more fully the metabolism of the α-aminoadipic acid in these important fermentations.

The reports on the presence of the tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine and isopenicillin N in *P. chrysogenum* along with the cell-free research from ABRAHAM's laboratory have led most

* The lactam has been found in *Cephalosporium* sp. in early biosynthetic studies on the formation of cephalosporin C[4].

** We are indebted to Dr. M. CRON for the rotation data.

workers to conclude that a penicillin N and/or isopenicillin N is the precursor to all hydrophobic penicillins. The isolation of significant amounts of penicillin V and the 6-oxo-piperidine-2-carboxylic acid from the same fermentation provide further evidence for the involvement of the tripeptide and a δ-(α-aminoadipyl) penicillanic acid in penicillin V production as well as indicate the metabolic fate of the other segment of the precursor.

References

1) GREENSTEIN, J. P. & WINTZ: Chemistry of the amino acids. Vol. 3, p. 2411, John Wiley and Sons, Inc., New York, 1961
2) LAM, S. & E. GRUSHKA: Reversed phase liquid chromatography of phenacyl esters of some natural penicillins. J. Liq. Chromat. 1: 33~34, 1978
3) a) JOHNSON, D. A. & G. A. HARDCASTLE: Reaction of 6-aminopenicillanic acid with carbon dioxide. J. Am. Chem. Soc. 83: 3534, 1961
b) BALLIO, A.; E. B. CHAIN, F. DENTICE DI ACADIA, M. MAURI, K. RAVER, M. J. SCHLESINGER & S. SCHLESINGER: Identification of a compound related to 6-aminopenicillanic acid isolated from culture media of *Penicillium chrysogenum*. Nature 191: 909, 1961
c) BATCHELOR, F. R.; D. GAZZARD & J. H. C. NAYLER: Action of carbon dioxide on 6-aminopenicillinic acid. Nature 191: 910, 1961
4) TROWN, P. W.; B. SMITH & E. P. ABRAHAM: Biosynthesis of cephalosporin C from amino acids. Biochem. J. 86: 284~291, 1963
5) LAKE, J. R.: Chemistry of peptides related to penicillins and cephalosporins. Dissertation, University Wisconsin–Madison, 1974
6) LODER, P. B.: Selected aspects of the biosynthesis of penicillins and cephalosporins. Post. Hig. I. Med. Dosw. 26: 493~500, 1972
7) FAWCETT, P. A.; J. J. USHER & E. P. ABRAHAM: Behaviour of tritium-labelled isopenicillin N and 6-aminopenicillanic acid as potential penicillin precursors in an extract of *Penicillium chrysogenum*. Biochem. J. 151: 741~746, 1975
8) MEISTER, A. & S. S. TATE: Glutathione and related γ-glutamyl compounds: Biosynthesis and utilization. Ann. Rev. Biochem. E. E. SNELL, P. D. BOYER, A. MEISTER & C. C. RICHARDSON eds. 45: 559~604, Annual Reviews, Palo Alto, Calif. 1976
9) FRIEDRICH, C. G. & A. L. DEMAIN: Uptake and metabolism of α-aminoadipic acid by *Penicillium chrysogenum* Wis 54–1255. Arch. Microbiol. 119: 43~47, 1978