J. Pharm. Pharmacol. 1986, 38: 913–917
Communicated August, 26, 1986

COMMUNICATIONS

N-Formylpenicillamine and penicillamine as degradation products of penicillins in solution

A. E. BIRD*, K. R. JENNINGS, A. C. MARSHALL, *Beecham Pharmaceuticals Research Division, Chemotherapeutic Research Centre, Brockham Park, Betchworth, Surrey RH3 7AJ, UK*

The degradation of several penicillins in unbuffered aqueous solution produces *N*-formylpenicillamine, in some cases in high yield. Very little or no penicillamine is formed under these conditions. *N*-Formylpenicillamine was produced from benzylpenicillin at pH values between 2·5 and 7, with a maximum yield of 30% at pH 5, whereas penicillamine was produced only at pH 5 or below in a yield of less than 1%. Benzylpenicillenic acid at pH 5 gave a 20% yield of *N*-formylpenicillamine and no penicillamine whereas benzylpenicilloic, penilloic and penillic acids gave no *N*-formylpenicillamine and a small amount of penicillamine.

N-Formylpenicillamine (IX) has been reported as a product of the degradation of benzylpenicillin (Hitomi 1959; Ueno et al 1984) and methicillin (Johnson & Panatta 1964) in aqueous solution and also as a product of enzymic reaction between benzylpenicillin or phenoxymethylpenicillin and carboxypeptidase or transpeptidase enzymes (Frere et al 1976; Adriaens et al 1978). However, many papers (Blaha et al 1976 and references therein; Degelaen et al 1979 and references therein; Kessler et al 1981, 1983; Mitsumori et al 1977) on the degradation of benzylpenicillin in solution make no mention of this potential product and there is no information in the literature on the extent of formation of *N*-formylpenicillamine or whether it is formed on degradation of penicillins other than methicillin and benzylpenicillin. These omissions are corrected in this paper, which includes details of a high performance liquid chromatographic method for determination of *N*-formylpenicillamine in solutions of penicillins. The method also detects the better known penicillin degradation product, penicillamine, levels of which were determined because little quantitative information about its extent of formation from penicillins is available in the literature. Furthermore, some studies relevant to the route of the formation of *N*-formylpenicillamine are reported.

* Correspondence.

Materials and methods

Materials and reagents. Benzylpenicillin, phenoxymethylpenicillin, and mezlocillin were obtained from Glaxo, Lilly and Bayer, respectively. Piperacillin was synthesized by standard methods. The other penicillins were Beecham commercial samples. Penicillamine and benzylpenicillenic acid were purchased from Aldrich and Sigma, respectively. Benzylpenicilloic and benzylpenilloic acids were made by the method of Munro et al (1978), benzylpenillic acid by the method of Cook (1949) and *N*-formylpenicillamine by the method of Adriaens et al (1978). Methanol and formic acid were reagent grade.

EDTA solution. Ethylenediaminetetraacetic acid disodium salt (10 mg) (reagent grade) was dissolved in 100 ml of 0·05 M pH 8·0 sodium phosphate buffer.

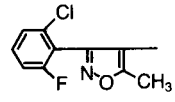
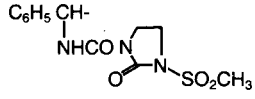
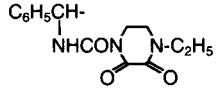
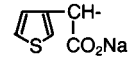
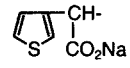
DTNB solution. 5,5-Dithiobis (2-nitrobenzoic acid). (2·0 mg) (BDH) was dissolved in 20 ml of 0·05 M pH 8·0 sodium phosphate buffer.

Buffers. pH 2·5, 0·01 M sodium phosphate. pH 4·0, 0·1 M sodium formate. pH 5·0, 0·1 M sodium acetate. pH 6·0, 0·1 M 2-(*N*-morpholino)ethane sulphonic acid. pH 7·0, 0·1 M 3-(*N*-morpholino)propane sulphonic acid.

Degradation studies. Solutions of the penicillin (2% m/v) in water or buffer were kept at 37 °C and samples taken at various times for determination of the *N*-formylpenicillamine and penicillamine contents, and pH. Solutions of benzylpenicilloic acid (10% m/v), benzylpenilloic acid (0·5% m/v), benzylpenillic acid (1% m/v) and benzylpenicillenic acid (0·05% m/v) in pH 5·0 acetate buffer at 37 °C were investigated similarly.

High performance liquid chromatography. HPLC was carried out with a Waters model 6000 pump, a prepacked Waters MicroBondapak C18 column fitted with a 20 µl loop injector and a variable wavelength ultraviolet detector (LDC Spectromonitor III) set at 330 nm 0·10 aufs. The system was operated at ambient temper-

Table 1. Formation of *N*-formylpenicillamine and penicillamine in 2% aqueous solutions of penicillins at 37 °C for 7 days.

Penicillin	R in structure III	*N*-formylpenicillamine Max yield (%)	*N*-formylpenicillamine Time to max (days)	Penicillamine yield (%) after 7 days	pH Initial	pH 7 days
Amoxycillin[a]	4-$OHC_6H_4CH(NH_2)$-	0·1	1	nd <0·1	7·3	6·6
Benzylpenicillin	$C_6H_5CH_2$-	15	4	nd <0·7	5·8	4·7[b]
Carbenicillin	$C_6H_5CH(CO_2Na)$-	19	7	nd <0·4	6·3	6·0
Flucloxacillin		35	7	0·9	5·2	4·3
Methicillin	2,6$(OCH_3)_2C_6H_3$-	46	2	0·7	5·9	3·6
Mezlocillin[a]		0·2	5	nd <0·1	7·1	6·0
Phenoxymethypenicillin	$C_6H_5OCH_2$-	3	6	0·1	6·3	5·1
Piperacillin[a]		nd <0·1	–	nd <0·1	6·9	6·3
Temocillin[c]		7	7	nd <0·2	7·7	7·5[d]
Ticarcillin		10	7	nd <0·2	7·5	6·4

nd = not detected.

Solutions were assayed after 1, 2, 3, 4 and 7 days except for carbenicillin, mezlocillin and phenoxymethylpenicillin which were 1, 2, 5, 6 and 7 days and piperacillin and ticarcillin which were 3, 4 and 7 days.

[a] Initial pH adjusted with NaOH.

[b] pH 4 after 1 day, then increasing.

[c] A 6α-methoxy 6β-aminopenicillanic acid derivative.

[d] pH 6·9 after 3 days, then increasing.

ature with a mobile phase of methanol – 0·1 M formic acid adjusted to pH 4·0 with sodium hydroxide (3 + 7 v/v) at a flow rate of 1·0 ml min^{-1}.

N-Formylpenicillamine and penicillamine were derivatized by reaction with DTNB and the product chromatographed. The method is based on that of Kuwata et al (1982) but with modifications to the mobile phase to give satisfactory resolution and retention times for the derivatives of *N*-formylpenicillamine and penicillamine. Standard solutions of these compounds were prepared containing an accurately known concentration of about 10 μg ml^{-1} in EDTA solution. 1·0 ml was mixed with 1·0 ml DTNB solution and allowed to react for 5 min at room temperature (20 °C) before chromatography. Samples of the degradation study solutions were diluted with EDTA solution as appropriate before reaction with DTNB as for the standards. Under the HPLC conditions given above the derivatives of penicillamine and *N*-formylpenicillamine elute after about 5 and 7 min, respectively, and unreacted DTNB elutes after about 10 min. Some samples of DTNB contain impurities which elute close to the penicillamine derivative peak.

Results and discussion

The extent of formation of *N*-formylpenicillamine and penicillamine in unbuffered solutions of various penicillins kept at 37 °C for 7 days is shown in Table 1, together with the initial and final pH values. The yield is given as a percentage of the theoretical for a 1 to 1 mole conversion. In all cases where measurement of *N*-formylpenicillamine was made at times after the maximum was reached, no significant change in yield was observed.

A very variable extent of formation of *N*-formylpenicillamine is seen among the different penicillins, ranging

from negligible amounts produced by the amino and ureido compounds amoxycillin, piperacillin and mezlocillin up to yields of 46 and 35% from methicillin and flucloxacillin, respectively. It is noteworthy that the yield from the 6-methoxy compound, temocillin, is similar to that from the analogous penicillin, ticarcillin. This is in marked contrast to the effect of a 6-formamido substituent in an acylureidopenicillin, which results in rapid and virtually complete conversion of the compound to *N*-formylpenicillamine (Cutmore et al 1986). Penicillamine was not detected at any time in most of the solutions listed in Table 1 and where it was seen the yield was no more than 1%. As expected, the pH of the solutions decreased on ageing, although in some cases an initial decrease was followed by a gradual increase, presumably due to decarboxylation of initial degradation products.

Solution pH affects the amount of *N*-formylpenicillamine produced from benzylpenicillin, as shown by the results in Table 2. A maximum rate and extent of conversion occurred at pH 5, where the yield increased steadily during the four days of observation. At pH 6 the conversion also increased throughout the observation period but at pH 4 the maximum stable yield of 8% was reached after only two days and at pH 2·5 the yield after 5 h decreased slowly in subsequent measurements. In these experiments penicillamine was detected in the pH 2·5 and pH 4 buffers at a low level and in the pH5 buffer at a very low trace level. Kessler et al (1981) have reported the formation of penicillamine in pH 2·5

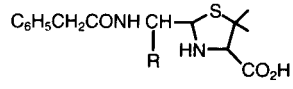
Table 2. Formation of *N*-formylpenicillamine and penicillamine in 2% buffered solutions of benzylpenicillin at 37 °C. Solutions were assayed after 1, 2, 3 and 4 days at pH 2·5, 4 and 5 and after 1, 2, 5 and 6 days at pH 6 and 7.

	N-Formylpenicillamine		Penicillamine
Buffer pH	Max yield (%)	Time for max (days)	Yield (%)
2·5	1·6[a]	0·2	0·8[b]
4·0	8	2	0·4
5·0	30	4	tr < 0·3
6·0	14	5	nd < 0·3
7·0	0·2	5	nd < 0·1

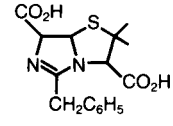
nd = not detected; tr = trace.
[a] Decreased to 1·2% after 1 and 2 days, 0·9% after 3 and 4 days.
[b] After 1 day; 0·5% after 2 days, 0·4% after 3 and 4 days.

phosphate in connection with studies of the degradation pathway of benzylpenicillin. Our results show that *N*-formylpenicillamine is a more important degradation product than penicillamine under these conditions, although the extent of formation of both is small.

In the original publication on the formation of *N*-formylpenicillamine in benzylpenicillin solutions, Hitomi (1959) stated in the English summary that benzylpenicilloic acid hydrolyses to give *N*-formylpenicillamine, but in the text he states that benzylpenicillenic acid was found to give *N*-formylpenicillamine. We have investigated the formation of *N*-formylpenicillamine at pH 5 in solutions of four important degradation products of benzylpenicillin, both to clarify the confusion in the Hitomi paper and to assist in deducing the route of conversion of the penicillin to *N*-formylpenicillamine. The results in Table 3 show that the penicilloic (I, R = CO_2H), penilloic (I, R = H) and penillic (II) acids of benzylpenicillin produce a small amount of penicillamine and no *N*-formylpenicillamine. By contrast, *N*-formylpenicillamine is rapidly produced from benzylpenicillenic acid (V), which produces no penicillamine. This accords with the observation of Johnson & Panatta (1964) that *N*-formylpenicillamine is produced by degradation of the penicillenic acid derived from methicillin. Under the conditions of our experiment the



I



II

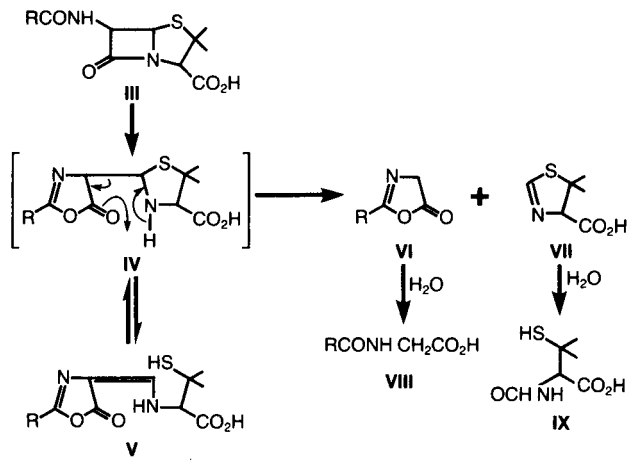
benzylpenicillenic acid was completely decomposed after about 0·75 h, the time at which the maximum yield (20%) of *N*-formylpenicillamine was first observed. The nature of the other degradation products was not investigated but conversion to the penicilloic and penillic acids are reactions known to occur easily (Longridge & Timms 1971).

The fact that *N*-formylpenicillamine is produced from penicillenic acids as well as from penicillins, but not from three other penicillin degradation products studied here, is consistent with its formation from the thiazoline (VII) produced by fragmentation of the unstable

Table 3. Formation of *N*-formylpenicillamine and penicillamine in pH 5 buffered solutions of benzylpenicillin and its degradation products at 37 °C. Solutions were assayed after 1, 2, 3, 4 and 5 h (benzylpenicilloic also after 6 h) except for benzylpenicillenic acid which was 0·25, 0·5, 0·75, 1, 1·25, 1·5, 1·75 and 2·5 h.

Compound and concn	*N*-Formylpenicillamine yield (%) at end of experiment	Penicillamine yield (%) at end of experiment
Benzylpenicillin, 2%	4·5	nd <0·03
Benzylpenicilloic acid, 10%	nd <0·02	1·1
Benzylpenilloic acid, 0·5%	nd <0·01	0·1
Benzylpenillic acid, 1%	nd <0·03	0·2
Benzylpenicillenic acid, 0·05%	20[a]	nd< 0·2

nd = not detected.
[a] 20% after 0·75 h and constant thereafter.



oxazolone-thiazolidine, IV (See Scheme.) This mechanism for formation of VIII (R = $C_6H_5CH_2$) and VII from IV (R = $C_6H_5CH_2$) was postulated by Hammarstrom & Strominger (1975) for the reaction of benzylpenicillin with a carboxypeptidase enzyme. Both VI (R = $C_6H_5CH_2$) and VII are known to undergo rapid hydrolysis in aqueous solution to give phenaceturic acid (VIII, R = $C_6H_5CH_2$) (Jansen & Robinson 1967) and *N*-formylpenicillamine (IX) (Adriaens et al 1978), respectively. The co-production of phenaceturic acid and 2,6-dimethoxyhippuric acid (VIII, R = 2,6$(OCH_3)_2$. C_6H_3) with *N*-formylpenicillamine on degradation of aqueous solutions of benzylpenicillin (Hitomi 1959) and methicillin (Johnson & Panatta 1964), respectively, supports the postulated mechanism shown in the Scheme. The ease and extent of formation of IV from penicillins will be dependent on the electronegativity of the R group, with the conversion being promoted by electropositive groups (Bundgaard 1980). This, and the possible effect of R on the mode of degradation of IV, could account for the varying extent of formation of *N*-formylpenicillamine from the different penicillins. The relative electronegativity of R for all the penicillins studied here, is not available, but those which show little production of *N*-formylpenicillamine do have relatively electronegative R groups, in accordance with the postulate. Also, the highest yields of *N*-formylpenicillin result from penicillins where an aromatic (methicillin) or heteroaromatic (flucloxacillin) group is directly attached to the side chain amide.

We thank Mr S. J. Mansey and Mr K. Lynch for technical assistance, Dr J. H. C. Nayler for discussions and chemists in the Development Chemistry Unit for preparation of compounds.

REFERENCES

Adriaens, P., Meesschaert, B., Frere, J.-M., Vanderhaeghe, H., Degelaen, J., Ghuysen, J.-M., Eyssen, H. (1978) J. Biol. Chem. 253: 3660–3665

Blaha, J. M., Knevel, A. M., Kessler, D. P., Mincy, J. W., Hem, S. L. (1976) J. Pharm. Sci. 65: 1165–1170

Bundgaard, H. (1980) Arch. Pharm. Chemi. Sci. Ed. 8: 161–180

Cook, A. H. (1949) in: Clarke, H. T., Johnson, J. R., Robinson, R. (eds) The Chemistry of Penicillin, Princeton Univ. Press, Princeton N.J., pp. 126–127

Cutmore, E. A., Guest, A. W., Hatto, J. D. I., Smale, T. C., Stachulski, A. V., Tyler, J. W. (1986) J. Chem. Soc. Chem. Comm. in press

Degelaen, J. P., Loukas, S. L., Feeney, J., Roberts, G. C. K., Burgen, A. S. V. (1979) J. Chem. Soc. Perkin II, 86–90

Frere, J.-M., Ghuysen, J.-M., Vanderhaeghe, H., Adriaens, P., Degelaen, J., DeGraeve, J. (1976) Nature 260: 451–454

Hammarstrom, S., Strominger, J. L. (1975) Proc. Nat. Sci. USA 72: 3463–3467

Hitomi, H. (1959) Yakugaku Zasshi 79: 1600–1606

Jansen, A. B. A., Robinson, R. (1967) Monatsh. Chem. 98: 1017–1026

Johnson, D. A., Panatta, C. A. (1964) J. Org. Chem. 29: 1826–1830

Kessler, D. P., Ghebre-Sellassie, I., Knevel, A. M., Hem, S. L. (1981) J. Chem. Soc. Perkin II: 1247–1251

Kessler, D. P., Cushman, M., Ghebre-Sellassie, I., Knevel, A. M., Hem, S. L. (1983) Ibid. 1699–1703

Kuwata, K., Uebori, M., Yamada, K., Yamazaki, Y. (1982) Anal. Chem. 54: 1082–1087

Longridge, J. L., Timms, D. (1971) J. Chem. Soc. (B): 852–857

Mitsumori, F., Arata, Y., Fujiwara, S., Muranaka, M., Horiuichi, I. (1977) Bull. Chem. Soc. Japan 50: 3164–3166

Munro, A. C., Chainey, M. G., Woroniecki, S. R. (1978) J. Pharm. Sci. 67: 1187–1204

Ueno, H., Nishikawa, M., Suzuki, S., Muranaka, M. (1984) J. Chromatog. 288: 117–126