Synthesis of (β-methyl^{3}H)-6β-iodopenicillanic acid

Fabien De Meester*, Jean-Marie Frère*", Jean-Louis Piette**
and Hubert Vanderhaeghe+

Summary

A rapid and easy synthesis of tritium-labelled β-iodopenicillanate, an efficient inactivator of serine β-lactamase, is described. This method involves only three reaction steps after the introduction of the label in the molecule with an overall yield of 16 %.

Key words : labelled β-iodopenicillanate, β-lactamase, penicillin sulphoxide.

Introduction

During the last few years, β-halogenopenicillanates have been widely utilized as specific and efficient inactivators of various β-lactamases (1-10). In several cases, these reagents were found to irreversibly react with a serine residue, essential for the enzymatic activity (8,11-12). This reaction probably involved the formation of a "normal" acyl enzyme intermediate (1, where E is the enzyme) which could rearrange into a more stable α-β unsaturated ester (2), a reaction accompanied by the elimination of the iodine atom.

1 2

* Laboratoire d'Enzymologie et Service de Microbiologie and
** Service de Chimie organique, Institut de Chimie, B6,
Université de Liège, B-4000 Sart Tilman, Liège, Belgium.
+ Rega Instituut, Katholieke Universiteit te Leuven,
Minderbroederstraat, 10, B-3000 Leuven, Belgium.
" To whom correspondence should be addressed.

0362-4803/85/050415-11$01.10

Received July 11, 1984
Revised September 30, 1984

Studies of the interaction between enzymic active sites and inactivators are usually greatly facilitated by the availability of a radiolabelled inactivator. Short of total synthesis or biosynthesis, labelling in the 2β-methyl group with ^{2}H or ^{3}H could be performed by heating a penicillin sulfoxide in the presence of deuterated or tritiated water (13-15). This procedure was utilized by Usher et al. (16) who prepared tritiated 6-aminopenicillanic acid from tritiated phenoxymethylpenicillin sulfoxide. This tritiated 6-aminopenicillanic acid was then transformed into 6α-bromopenicillanic acid by Knott-Hunziker et al. (17) who finally obtained the 6-β isomer by epimerizing 6α-bromopenicillanate at pH 9.2 . The yield of this latter step was low (5 %).

This method involved a large number of reaction steps utilizing labelled compounds. In a first attempt to reduce this number, tritium was introduced in the sulfoxide of the p-methoxybenzyl ester of α-bromopenicillanic acid (18) which was then reduced, deprotected and epimerized (three steps). However, the yield of the epimerization step was rather poor (5 %). In this paper, we describe a new method which allows an easy preparation of tritiated β-iodopenicillanic acid, involving only three steps after the introduction of the label.

Material and Methods

Unlabelled β-iodopenicillanic acid and 6-aminopenicillanic acid were gifts, respectively, from Dr. J. Kemp, Pfizer Central Research, Sandwich, U.K. and from Dr. M. Januszewski, Beecham S.A., Heppignies, Belgium. Tritiated water (5 Ci/ml) was from the Radiochemical Center, Amersham, U.K., and all the reagents for organic synthesis were from Janssen Pharmaceutica, Beerse, Belgium. The β-lactamase of Bacillus licheniformis was purified as described by Thatcher (19). UV spectra were recorded using a Beckman DU8 spectrophotometer.

Radioactivity measurements were performed by liquid scintillation using a Packard Tri-Carb spectrometer or a Packard 7201 radiochromatogram scanner, and NMR and Infra-red spectra were recorded using a 60 MHz Varian EM360L or a Perkin Elmer 1320 spectrometer, respectively. Silica-gel for column chromatography (Kieselgel 60 , 0.06 - 0.2 mm , Merck) was deactivated by suspending in water and evaporating the solvent under vacuum. Thin-layer chromatography was performed using Kieselgel 60 F_{254}, D.C. Alufolien, Merck. Solvent A was $CHCl_3$:CH_3OH:CH_3COOH 88/10/2 v/v/v .

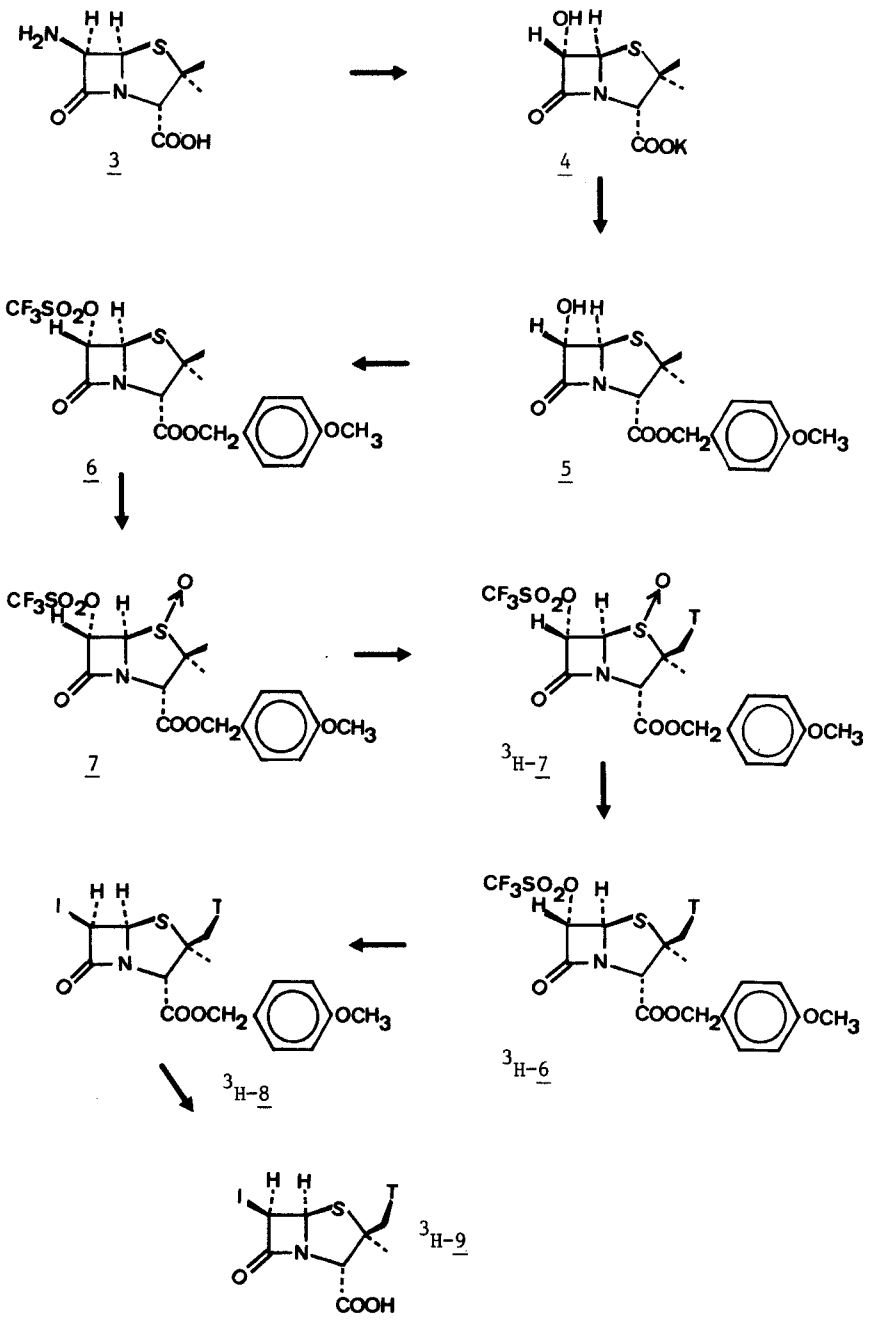


Fig. 1. Synthesis strategy.

Results

The synthesis strategy is depicted by Fig. 1. The starting material was the commercial 6β-aminopenicillanic acid (3) which was diazotised in the presence of perchloric acid. The resulting 6α-hydroxy-penicillanic acid potassium salt (4) was successively transformed into the p-methoxybenzylester (5) and triflate derivative (6). The oxidation to S-sulphoxide (7) was carried out with m-chloroperbenzoic acid with a yield of 90 %. The label was introduced by exchange between $^{3}H_2O$ and 7. The tritiated compound was isolated, reduced with PBr_3, iodine was introduced by a SN_2 reaction on C_6 and the ester was hydrolysed with trifluoroacetic acid. The three steps performed after introduction of the label exhibited an overall yield of 16 %. The overall yield of the four steps performed before the introduction of the label was 12 %.

The purity of the compound was checked by thin-layer chromatography. Scanning of the chromatogram revealed the presence of only one peak, whose position corresponded to that of standard β-iodopenicillanic acid (Fig. 2). At that stage, the specific radioactivity was 2.4 mCi/mmole.

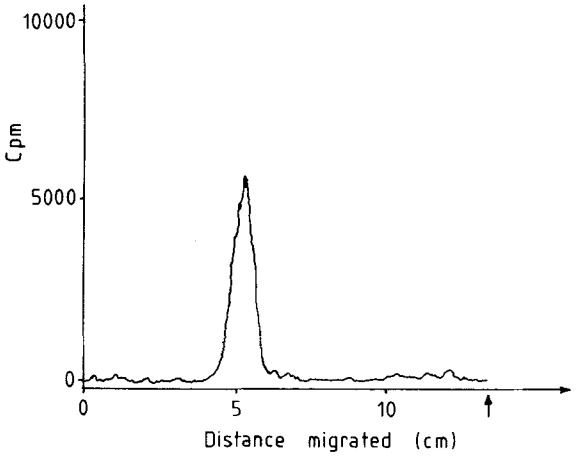


Fig. 2. Scanning of the chromatogram after elution with solvent A. Conditions were as follows : scan rate : 1 cm min^{-1}, time constant : 3 s, slidwidth : 2 mm. The arrow indicates the position of the solvent front. The R_f of the radioactive spot was 0.40 and exactly corresponded to that of authentic β-iodopenicillanic acid. The position of this compound was revealed by heating the plate at 110° for 30 min : a brown spot appeared, probably due to the formation of iodine.

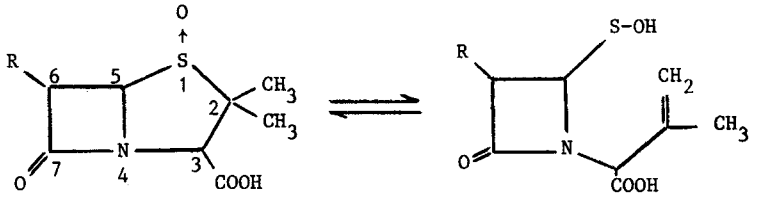
The tritiated β-iodopenicillanic acid was diluted 78.2 fold with the unlabelled compound and two successive recrystallizations were performed from a mixture of CH_2Cl_2 and methanol. After each crystallization, the specific activity of a sample was determined and a value of 0.0289 mCi/mmole was found in both cases, corresponding to 2.26 mCi/mmole for the undiluted material.

Finally, the radioactive β-iodopenicillanic acid was used to label the active site of the Bacillus licheniformis β-lactamase : 440 µg of enzyme (15.6 nmoles) and 251 nmoles of β-iodopenicillanate were incubated for 20 min at 30° in a total volume of 500 µl , 100 mM Hepes buffer, pH 8.0 . The solution was extensively dialyzed against the same buffer. The UV spectrum of the sample indicated the presence of the dihydrothiazine chromophore usually observed after inactivation of serine β-lactamases by β-halogenopenicillanates (12,20). The specific activity of the enzyme-dihydrothiazine adduct was 2.44 mCi/mmole, corresponding to an inactivator/enzyme ratio of 1.08 . This experiment also demonstrated the stability of the labelling. Moreover, the specific activity of the tritiated compound has not changed after a 6-month storage period (4°C, in the dark and in the presence of a dessicating agent).

Discussion

In this communication, we have described an easy method for the synthesis of tritium-labelled β-iodopenicillanic acid. Tritium was introduced in the molecule by exchange with tritiated water at the level of the sulphoxide of the methoxybenzylester of 6β-trifluoromethylsulphonyloxypenicillanic acid. From this compound, (β-methyl^{3}H)-6β-iodopenicillanic acid was obtained in only three steps with a yield of 16 % , a result which represented a considerable improvement over the previously described methods.

The position of the tritium was not determined in the present work. In an experiment where D_2O was substituted for THO in the exchange reaction, the intensity of the β-methyl band was decreased by 25 % in the NMR spectrum. The exchange reaction at the level of the S-sulfoxide (10) is thought (13) to proceed via the sulfenic acid (11) :



10 11

Since the position of the label was clearly established in the same exchange reaction when the molecule only differed by the nature of substituent R (16), it seemed safe to assume that the label was in the β-methyl group. The label remained stable upon storage and during the rearrangement of the acyl-enzyme formed after reaction with a β-lactamase into the dihydrothiazine chromophore.

It is interesting to note that compound ^{3}H-6 in which the trifluoromethylsulphonyloxy group can easily be displaced by a nucleophile in a SN_2 reaction can serve as a starting point for the easy synthesis of a wide variety of tritium-labelled 6-β substituted derivatives of penicillanic acid.

The specific activity of the final compound is quite low. It should be noted that, however, while it is relatively easy to label a penicillin in the side chain on C_6, it is quite difficult to obtain a compound labelled in the bicyclic system. Apart from the exchange procedure utilized in the present study, the only possible methods for introducing a tritium or ^{14}C atom in this part of the molecules would be total synthesis or biosynthesis, which would require numerous reaction steps in the first case or would produce very low yields in the second. A higher specific activity could be obtained by increasing the amount or specific activity of THO in the exchange step but specialized equipment for handling large activities would then be necessary, and the specific activity obtained in the present work is quite sufficient for enzyme active site labelling experiments.

Experimental

6α-Hydroxypenicillanic acid potassium salt (4) [adapted from (21)]

A solution of 9.5 g (123 mmoles) of sodium nitrite in 120 ml of water was added dropwise to a cooled (5°) solution of 20.5 g (95 mmoles) of 6β-aminopenicillanic acid (3) in 260 ml of 1 N perchloric acid. To avoid oxidation of the diazonium salt intermediate, this step was performed under inert atmosphere and in the dark. The yellow foaming solution was saturated with sodium chloride and extracted with diethyl ether. The organic layer was washed with a cold saturated sodium chloride solution, dried over magnesium sulphate and the solvent evaporated. The oil thus obtained was dissolved in dichloromethane (100 ml) and water was added (100 ml). The mixture was cooled to 0° and the pH adjusted to 6.8 by addition of diluted potassium hydroxide. The residue obtained upon

evaporation of the aqueous layer was triturated with dry acetone, yielding 6.75 g (26.5 mmoles) of the solid 6α-hydroxypenicillanic acid potassium salt (4).

Yield : 27 % .

NMR (D_2O) : 1.60 (s, 3-H), 1.66 (s, 3-H), 4.37 (s, 1-H), 4.93 (d, 1-H : J = 1.2 Hz), 5.32 (d, 1-H : J = 1.2 Hz).

6α-Hydroxypenicillanic acid 4-methoxybenzyl ester (5)

p-Methoxybenzylbromide [20 g ; 99.5 mmoles, prepared as in (22)] was added to a stirred solution of 4 (25 g ; 98 mmoles) in N,N-dimethylformamide (800 ml). The mixture was stirred at room temperature for 17 hours and then partitioned between water (1 l) and ethyl acetate (1 l). The organic layer was separated, successively washed with water (2 × 500 ml), saturated sodium bicarbonate (500 ml), brine (500 ml) and dried over magnesium sulphate. Evaporation of the solvent gave 29.4 g (87.2 mmoles) of 6α-hydroxypenicillanic acid 4-methoxybenzyl ester (5) as an oil.

Yield : 88 % representing a considerable improvement over the 13 % yield previously described for a similar reaction (21).

NMR ($CDCl_3$) : 1.35 (s, 3-H), 1.50 (s, 3-H), 3.78 (s, 3-H), 4.42 (s, 1-H), 4.87 (d, 1-H : J = 1.5 Hz), 5.09 (s, 2-H), 5.23 (d, 1-H : J = 1.5 Hz), 6.84 (d, 2-H : J = 8.8 Hz), 7.27 (d, 2-H : J = 8.8 Hz).

6α-Trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (6)

This was done as in (21) on 6.6 g (20 mmoles) of 5 .

Yield : 54 % .

m.p. : 69-71° (litt : 69-71°).

NMR ($CDCl_3$) : 1.36 (s, 3-H), 1.53 (s, 3-H), 3.80 (s, 3-H), 4.52 (s, 1-H), 5.12 (s, 2-H), 5.48 (s, 2-H), 6.89 (d, 2-H : J = 8.7 Hz), 7.29 (d, 2-H : J = 8.7 Hz).

IR (film) : 1798, 1745 cm^{-1}.

6α-Trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (S)-sulphoxide (7)

A cooled solution (0°) of 6 (4.69 g ; 10 mmoles) in dichloromethane (100 ml) was treated dropwise with a solution of m-chloroperbenzoic acid (2.03 g ; 10 mmoles) in dichloromethane (100 ml) over a period of 30 min . The reaction mixture was stirred for 30 min at room temperature and diluted with dichloromethane (100 ml). The organic layer

was washed with 5 % potassium hydrogen carbonate (2 × 100 ml), with ice-water (2 × 100 ml) and dried over magnesium sulphate. Evaporation of the solvent gave a colourless oil which crystallised in diethylic ether to yield 4.36 g (9 mmoles) of the crystalline 6α-trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (S)-sulphoxide (7).

Yield : 90 %.

m.p. : 111-113°.

NMR ($CDCl_3$) : 1.07 (s, 3-H), 1.57 (s, 3-H), 3.74 (s, 3-H), 4.57 (s, 1-H), 5.2 (d, 2-H : J = 3.5 Hz), 5.27 (d, 1-H : J = 1.2 Hz), 5.73 (d, 1-H : J = 1.2 Hz), 6.9 (d, 2-H : J = 9 Hz), 7.37 (d, 2-H : J = 9 Hz).

IR : 1798,1755 cm^{-1}.

This step was an adaptation of the oxidation procedure described in (15) for 6α-bromopenicillanic acid 4-methoxybenzyl ester, which yielded the (S) isomer. Our NMR data indicated that the same isomer had been obtained in the present work.

(β-Methyl ^3H)-6α-trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (S)-sulphoxide (^3H-7)

Incorporation of tritium in penicillin sulphoxide has been described by Usher et al. (16). 7 (2.78 g ; 5.73 mmoles), benzene (110 ml) and ^3H_2O (0.1 ml ; 5.56 mmoles ; 500 mCi) were stirred at 80° for 3 hours in a flask fitted with a reflux condenser. The reaction mixture was dried over magnesium sulphate and the solvent evaporated to give 2.5 g (5.15 mmoles) of the pure crystalline (β-methyl ^3H)-6α-trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (S)-sulphoxide (^3H-7).

Yield : 89 %.

m.p. : 111-113°.

Specific radioactivity : 2.4 mCi/mmole.

(β-Methyl ^3H)-6α-trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (^3H-6)

To a cooled solution (0°) of ^3H-7 (2.5 g ; 5.15 mmoles) in N,N-dimethylformamide (66 ml) was added 1.9 ml of phosphorus tribromide (20.2 mmoles). The reaction mixture was stirred during 20 min at 0°, added with 5 % potassium hydrogen carbonate (200 ml) and extracted with ethyl acetate (3 × 200 ml). The organic layer was washed with water (2 × 400 ml) and dried over magnesium sulphate. After evaporation of the solvent, the colourless gum was triturated with pentane to give 2.2 g (4.68 mmoles) of

the crystalline (β-methyl^{3}H)-6α-trifluoromethylsulphonyloxypenicillanic acid 4-methoxybenzyl ester (^{3}H-6).

Yield : 90 %.

m.p. : 69-71°.

Specific radioactivity : 2.4 mCi/mmole.

This step was adapted from (18).

(β-Methyl^{3}H)-6β-iodopenicillanic acid 4-methoxybenzyl ester (^{3}H-8)

A mixture of ^{3}H-6 (2.2 g ; 4.68 mmoles), sodium iodide (5.5 g ; 36.7 mmoles) and acetone (38 ml) was stirred at room temperature for 46 hours. The resulting mixture was concentrated to 5 ml , diluted with water (80 ml) and extracted with diethyl ether (100 ml). The ether extract was washed with water (100 ml), dried over magnesium sulphate and evaporated to yield 1.9 g (4.25 mmoles) of (β-methyl^{3}H)-6β-iodopenicillanic acid 4-methoxybenzyl ester (^{3}H-8) as a pale yellow gum. Crystals were obtained from CCl_4 with a very poor yield. The synthesis was continued with the gum.

Yield : 90 %.

m.p. (of the crystals) : 66-70° (litt : 68-70°).

NMR ($CDCl_3$) : 1.39 (s, 3-H), 1.69 (s, 3-H), 3.86 (s, 3-H), 4.56 (s, 1-H), 5.18 (s, 2-H), 5.43 (d, 1-H : J = 4.2 Hz), 5.65 (d, 1-H : J = 4.2 Hz), 6.95 (d, 2-H : J = 9.2 Hz), 7.36 (d, 2-H : J = 9.2 Hz).

IR : 1805,1745 cm^{-1}.

This step was adapted from (2).

(β-Methyl^{3}H)-6β-iodopenicillanic acid (^{3}H-9)

Trifluoroacetic acid (9.5 ml) was added to a solution of ^{3}H-8 (1.9 g ; 4.25 mmoles) in dichloromethane (95 ml). The solution was stirred at room temperature for 30 min and then poured in benzene (950 ml). After evaporation of the solvent, the residue was chromatographed on a column of deactivated silica (60 g) eluted with a 1:3 mixture of ethyl acetate and petroleum ether (BP60/80°). The fractions containing the product were combined, dried over magnesium sulphate and evaporated to a low volume. The crystalline precipitate was collected by filtration, washed with a 1:1 mixture of dichloromethane and pentane, and dried to yield 0.28 g (0.856 mmoles) of (β-methyl^{3}H)-6β-iodopenicillanic acid (^{3}H-9).

Yield : 20 %.

m.p. : 120° (DEC).

Specific radioactivity : 2.4 mCi/mmole.

NMR ($CDCl_3$) : 1.57 (s, 3-H), 1.74 (s, 3-H), 4.57 (s, 1-H), 5.39 (d, 1-H : J = 4 Hz), 5.65 (d, 1-H : J = 4 Hz), 8.95 (s, 1-H COOH).

IR : 1798,1715 cm^{-1}.

This step was adapted from (23).

Acknowledgements

The work was supported in part by the *Fonds de la Recherche Scientifique Médicale*, Brussels (contract n°3.4507.83), an *Action concertée* financed by the Belgian State (convention n°79/84-I1), and grants from the *Fonds National de la Recherche Scientifique* and the *Région wallonne* (*convention tripartite du 1.12.83*). We wish to thank Dr. M. Januszewski, Beecham S.A., Heppignies, Belgium and Dr. J. Kemp, Pfizer Central Research, Sandwich, U.K. for gifts of compounds.

References

1. Cartwright, S.J. and Fink, A.L. - FEBS Lett. 132:186 (1982).
2. Pratt, R.F. and Loosemore, M.J. - Proc. Natl. Acad. Sci. USA 75:4145 (1978).
3. Wise, R., Andrews, J.M. and Patel, N. - J. Antimicrob. Ag. Chemother. 7:531 (1981).
4. Moore, B.A. and Brammer, K.W. - Antimicrob. Ag. Chemother. 20:327 (1981).
5. Orlek, B.S., Sammes, P.G., Knott-Hunziker, V. and Waley, S.G. - J. Chem. Soc. Chem. Commun., 962-963 (1979).
6. Knott-Hunziker, V., Orlek, B.S., Sammes, P.G. and Waley, S.G. - Biochem. J. 187:797 (1980).
7. Loosemore, M.J., Cohen, S.A. and Pratt, R.F. - Biochemistry 19:3990 (1980).
8. Cohen, S.A. and Pratt, R.F. - Biochemistry 19:3996 (1980).
9. Orlek, B.S., Sammes, P.G., Knott-Hunziker, V. and Waley, S.G. - J. Chem. Soc. Perkin Trans. I, 2322-2329 (1980).
10. Frère, J.M., Dormans, C., Duyckaerts, C. and De Graeve, J. - Biochem. J. 207:437 (1982).
11. Knott-Hunziker, V., Waley, S.G., Orlek, B.S. and Sammes, P.G. - FEBS Lett. 99:59 (1979).
12. Joris, B., Dusart, J., Frère, J.M., Van Beeumen, J., Emanuel, E., Gagnon, J. and Waley, S.G. - Biochem. J. 223:271 (1984).
13. Cooper, R.D.G. - J. Amer. Chem. Soc. 92:5010 (1970).
14. Burton, D.H.R., Comer, F., Greig, D.G.T., Lucente, G., Sammes, P.G. and Underwood, W.G.E. - Chem. Commun, 1059-1060 (1970).

15. Meesschaert, B., Adriaens, P., Eyssen, H., Roets, E. and Vanderhaeghe, H.- J. Antibiotics 29:433 (1976).
16. Usher, J.J., Loder, B. and Abraham, E.P. - Biochem. J. 151:729 (1975).
17. Knott-Hunziker, V., Orlek, B.S., Sammes, P.G. and Waley, S.G. - Biochem. J. 177:365 (1979).
18. Claes, P., Thomis, J., Roets, E., Vanderhaeghe, H., De Meester, F. and Piette, J.L. - J. Antibiot., submitted.
19. Thatcher, D.R. - Methods Enzymol. 43:653 (1975).
20. Loosemore, M.J., Cohen, S.A. and Pratt, R.F. - Biochemistry 19:3990 (1980).
21. Hauser, D. and Sigg, H.P. - Helv. Chim. Acta 50:1327 (1967).
22. Lapworth, A. and Shoesmith, J.B. - J. Chem. Soc. CXXI:1397 (1922).
23. Kemp, J.E.G., Closier, M.D., Narayanaswami, S. and Stefaniak, M.H. - Tetrahedron Lett. 21:2991 (1980).