Tetrahedron Vol. 43, No. 18, pp. 4217 to 4220, 1987
Printed in Great Britain

0040-4020/87 $3.00+.00

AN EFFICIENT SUBSTITUTE FOR THE α-AMINOADIPOYL MOIETY OF δ-(L-α-AMINOADIPOYL)-L-CYSTEINYL-D-VALINE IN THE ENZYMATIC SYNTHESIS OF PENICILLINS

JACK E. BALDWIN,* ROBERT M. ADLINGTON, M. JAMES C. CRABBE, TAKASHI NOMOTO AND CHRISTOPHER J. SCHOFIELD

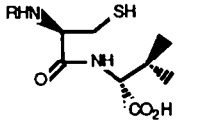
The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QY, U.K

(*Received in UK* 22 *June* 1987)

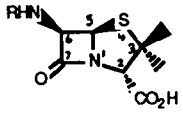
Abstract: meta-Carboxyphenylacetyl-L-cysteinyl-D-valine was shown to be a highly efficient substrate for Isopenicillin N Synthetase, with similar Michaelis constant and maximum velocity parameters to the natural substrate δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine.[1]

Studies in this laboratory have demonstrated that variations of the α-aminoadipoyl residue of the natural substrate, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) (1a) of Isopenicillin N Synthetase (IPNS) require a 6-carbon or equivalent chain, terminating in an acidic function, to be efficient substrates. Thus L- or D-α-aminoadipoyl-(1a), (1b) and adipoyl- (1c) L-cysteinyl-D-valines were effective substrates,[2] but the phenylacetyl-(1d) and phenoxyacetyl (1e) peptides were converted only at very slow rates into 2d and 2e, respectively.[3] Penicillins with such arylamido side chains possess potent antibacterial activity, hence their direct enzymatic synthesis, from tripeptides obviating the in vivo deacylation-reacylation process, is of considerable interest.

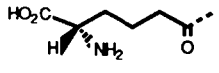
In order to increase the efficiency of the enzymatic synthesis of the arylamido penicillins, we hypothesised that the m-carboxyphenylacetyl moiety would represent a conformationally rigid form of the required transoid 6-carbon chain terminating in a carboxy function, and therefore provide an effective substitute.

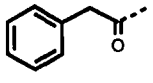


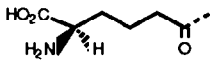
1

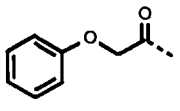


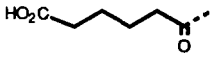
2

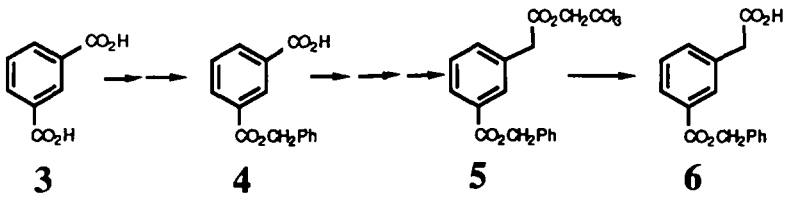
a;R= 

d;R= 

b;R= 

e;R= 

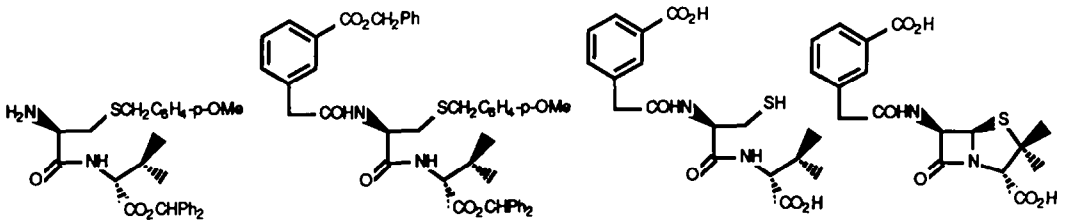
c;R= 



3 **4** **5** **6**

Scheme 1

Thus 3-(benzyloxycarbonyl)phenylacetic acid (6) was synthesised from isophthalic acid, via standard methodology as in Scheme 1, and coupled[4] with S-p-methoxybenzyl-L-cysteinyl-D-valine benzhydryl ester (7) to give the protected peptide (8). Deprotection (CH_2Cl_2, anisole, $AlCl_3$, 0-20°C)[5] gave the desired peptide (9).



7 **8** **9** **10**

Incubation of 9 with highly purified IPNS from Cephalosporin acremonium CO 728 and the required co-factors[6] gave, after purification by h.p.l.c., the m-carboxyphenylacetylpenam (10) in 70% yield(n.m.r. integration calibration against dioxan internal standard).

A comparison of the steady-state kinetic parameters K_m and V_{max} for the tripeptides (1a), (1d) and (9) with IPNS was carried out with a coupled enzyme assay utilizing β-lactamase 1 (from Bacillus cereus) and pH Stat titration[7] (Table 1). As V_{max} (= Kcat $[E]_o$) represents the lower limit on the rate constants for catalysis, these results suggest that the catalytic turnover of the m-carboxyphenylacetyl peptide (9) occurs much more rapidly [with similar magnitude to the natural substrate (1a)] than for the phenylacetyl tripeptide (1d). This is despite similar dissociation constants for the enzyme bound species (represented in the steady state by K_m) for 1d and 9.

Substrate	K_m (mM)	v_{max} (μmol min^{-1})
1a	0.16	1.6
1d	0.9	2.5×10^{-3}
9	0.8	0.8

Table 1

Recently we have shown that the m-carboxyphenylacetyl moiety is also an efficient substitute for the D-α-aminoadipoyl side chain of the natural substrate (penicillin N, 1b) for Deactoxy (DAOC)/Deacetyl (DAC) Cephalosporin C Synthetase, from C.acremonium CW19.[8] Thus both enzymes appear to show a preference for a transoid 6-carbon chain terminating in an acidic moiety. That IPNS accepts both L- and D-configured α-aminoadipoyl side chains whereas DAOC/DAC Synthetase accepts only the latter, possibly reflects the relative positioning of specific residues within each active site.[9]

Acknowledgements: We thank the S.E.R.C. and Eli Lilly & Co., for financial support.

GENERAL EXPERIMENTAL

Standard synthetic chemical procedures as previously reported were used.[10] Melting points were recorded on a Büchi 510 apparatus and are uncorrected. Infra red spectra were recorded on a Perkin Elmer 681 spectrophotometer. ^{1}H n.m.r. spectra were either recorded at 300 MHz on a Bruker WH300 spectrometer or at 500MHz on a Bruker AM500 spectrometer. Mass spectra in the electron impact, or chemical ionisation modes were recorded on a VG Micromass 16F spectrometer. Samples requiring desorption chemical ionisation or fast atom bombardment were run on VG Micromass 30F or ZAB 1F spectrometers.

H.p.l.c. of the crude incubation mixtures was carried out using a Waters M-6000 A pump, a UK-6 injector or a Rheodyne 7125 injector and a PYE Unicam LC3 UV detector and of the tripeptide substrate using dual Gilson 303 pumps, a Rheodyne 7125 injector and a Gilson holochrome set at 220 nm. The pH Stat was supplied by Radiometer, Copenhagen.

Isophthalic acid monobenzyl ester (4)

To a suspension of isophthalic acid (11.2 g, 0.1 mol) in MeOH (200 ml) and H_2O (10 ml) was added a solution of KOH (11.2 g, 0.2 mol) in MeOH (100 ml), and the mixture stirred for 15 hours. The solvent was removed in vacuo and dimethylformamide (250 ml) and benzyl bromide (13.5 ml, 0.11 mol) added. The mixture was heated for 2 hours at 100°C, cooled to 20°C and poured into aqueous sodium bicarbonate solution (10 g in 500 ml water), and extracted with ethyl acetate (3 x 100 ml). The aqueous layer was acidified to pH 1 (with conc. HCl), filtered and extracted with ethyl acetate (3 x 100 ml). The organic extracts were combined, washed with brine (200 ml), dried (Na_2SO_4) and evaporated to dryness. Chromatography [flash silica (ether/hexane)] gave 4 (3.73 g, 21%). TLC (ether/hexane, 1:1) Rf 0.35; ν_{max} ($CHCl_3$) 2700-3400 b (CO_2H), 1720 s (CO), 1700 s (CO) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 5.42 (2H, s, CH_2Ar), 7.30-7.50 (5H, m, Ar-H), 7.59 (1H, t, J 7.5 Hz, Ar-H), 8.32 (2 x 1H, 2 x d, J 7 Hz, Ar-H), 8.81 (1H, s, Ar-H).

2,2,2-Trichloroethyl-[m-benzyloxycarbonyl]phenyl acetate (5)

A solution of 4 (2.60 g, 10.2 mmol) in benzene (8 ml) and thionyl chloride (2.6 ml) was heated for 1 hour at 80°C. The reaction mixture was then evaporated to dryness and re-dissolved in benzene (20 ml), treated with an ethereal solution of diazomethane (excess) at 0°C, and stirred for 20 minutes. The reaction mixture was then evaporated to dryness and treated with Cl_3CCH_2OH (12 ml). The resultant mixture was warmed to 50-60°C and Ag_2O (ca 0.3 g) was added. After 2 hours Ag_2O (ca 0.3 g) was added and heating continued for 30 minutes, when further Ag_2O (ca 0.2 g) was added. After 30 minutes the reaction mixture was cooled, diluted with chloroform (30 ml), treated with charcoal (ca 0.5 g), filtered (celite) and evaporated to dryness. Chromatography [flash silica (benzene/40-60 petroleum ether, 1:1)] gave 5 (3.60 g, 88%). TLC (chloroform) Rf 0.5; ν_{max} ($CHCl_3$) 3020 m, 2960 w, 1755 s, 1740 s, 1610 w cm^{-1}; δ_H (300 MHz, $CDCl_3$) 3.83 (2H, s, $ArCH_2CO_2$), 4.76 (2H, s, CH_2CCl_3) 5.37 (2H, s, CH_2Ph), 7.30-7.60 (7H, m, Ar-H), 8.02-8.06 (2H, m, Ar-H); m/e (electron impact) 404/402/400 (M^+, 7%), 297 (14%), 295 (42%), 91 (100%).

m-(Benzyloxycarbonyl)phenyl acetic acid (6)

Zn dust (7.2 g, 0.11 mol), followed by 1 M aqueous potassium dihydrogen phosphate (7.2 ml) was added to a rapidly stirred solution of 5 (3.60 g, 9.0 mmol) in tetrahydrofuran (36 ml) at 20°C.[11] After 10 minutes further Zn (7.2 g, 0.11 mol) was added, and after 30 minutes the reaction mixture was filtered and evaporated to dryness. The residue was dissolved in chloroform (100 ml) and 2N hydrochloric acid (20 ml). The organic layer was separated and the aqueous layer further extracted with chloroform (2 x 100 ml). The organic extracts were combined, dried (Na_2SO_4) and evaporated to dryness. Chromatography [flash silica (ethyl acetate)] gave 6 (896 mg, 37%). TLC (ethyl acetate) Rf 0.35; m.p. 95-96°C (from ether); ν_{max} ($CHCl_3$) 2500-3400 b, 1715 s (CO) cm^{-1}; δ_H (300 MHz, $CDCl_3$) 3.72 (2H, s, CH_2CO_2H) 5.37 (2H, s, CO_2CH_2Ph), 7.30-7.55 (7H, m, Ar-H), 7.98-8.09 (2H, m, Ar-H); m/e (electron impact) 270 (M^+, 10%), 252 (67%), 163 (100%); Anal. [Found: C, 71.22%, H, 5.27%. Calcd. for $C_{16}H_{14}O_4$: C, 71.10%; H, 5.22%].

(m-Benzyloxycarbonylphenyl acetyl)-(S-p-methoxybenzyl-L-cysteinyl)-(D-valine benzhydryl ester) (8)

A solution of 6 (427 mg, 1.58 mmol), 1-ethoxycarbonyl-2-ethoxy-dihydroquinoline (390 mg, 1.58 mmol) and 7[13] (799 mg, 1.58 mmol) in dichloromethane (10 ml) were stirred under an inert atmosphere for 24 hours.[8] The solvent was removed in vacuo, the residue dissolved in ethyl acetate (80 ml), washed with sodium bicarbonate solution (50 ml), brine (50 ml), 10% citric acid solution (50 ml), brine (50 ml), dried (Na_2SO_4) and evaporated to dryness. Chromatography [flash silica (ethyl acetate/40-60 petroleum ether)] gave 8 (1.01 g, 84%). TLC (ethyl acetate, 40-60 petroleum ether, 1:1) Rf 0.4; m.p. 136-137°C (from ether); ν_{max} ($CHCl_3$) 3400 b, 3010 m, 2960 m, 1720 s, 1670 s, 1610 m, 1515 s, 1500 m cm^{-1}; δ_H (300 MHz, $CDCl_3$) 0.73, 0.85 (2 x 3H, 2 x d, J 7 Hz, $CHCH_3$) 2.20-2.25 [1H, m, $CH(CH_3)_2$], 2.58-2.86 (2H, 8 lines AB part of ABX system, $CHCH_2S$), 3.56, 3.69 (2 x 2H, 2 x

s, SCH_2Ph, $COCH_2Ar$), 3.76 (3H, s, OMe), 4.52 (1H, dd, J 7.5, 5.5 Hz, $CHCHCH_3$), 4.60-4.65 (1H, m, $CHCH_2S$), 5.35 (2H, s, CH_2Ph), 6.35, 6.70 [1H, 2 x d, J 7.5 Hz, and 8.5 Hz, NH (rotamers)], 6.83, 7.16 [1H, 2 x d, J 8 Hz and 8 Hz, NH (rotamers)], 6.91 (1H, s, $CHPh_2$), 7.28-7.48 (17H, m, Ar-H), 7.92-8.40 (6H, m, Ar-H); Anal. [Found: C, 71.12%; H, 6.02%; N, 3.59%. Calcd. for $C_{45}H_{46}N_2O_7S$: C, 71.22%; H, 6.11%; N, 3.69%].

m-Carboxyphenylacetyl-L-cysteinyl-D-valine (9)

To a solution of 8 (150 mg, 0.20 mmol) in dichloromethane (3 ml) and anisole (0.36 ml, 3.6 mmol), at 0°C, was added aluminium trichloride (2 ml of a 0.5 M solution in nitromethane). The mixture was stirred for 2 hours at 0°C and 3 hours at 20°C,[8] after which ethyl acetate (40 ml) was added. The organic solution was washed with 1N hydrochloric acid (25 ml) and extracted with 5% aqueous sodium bicarbonate (2 x 20 ml). The aqueous extract was washed with ethyl acetate (20 ml), acidified to pH 1 (with 2N hydrochloric acid) and extracted with ethyl acetate (3 x 25 ml). The organic extracts were combined, washed with brine, dried (Na_2SO_4) and evaporated to dryness to give 9 (70 mg, 93%). m.p. 196-197°C (decomp., recrystallised from acetone); ν_{max} (KBr disc) 3300 m, 2800-3300 b, 2680 w, 2560 w, 1730 s, 1700 s, 1650 s, 1605 s cm^{-1}; δ_H (300 MHz, CD_3OD) 0.89, 0.94 [2 x 3H, 2 x d, J 7 Hz, $CH(CH_3)_2$], 2.12-2.18 [1H, m, $CHCCH_3)_2$], 2.79-2.92 (2H, m, CH_2S), 3.67 (2H, s, CH_2Ar), 4.31 (1H, d, J 5.5 Hz, $CHCHCH_3$), 4.58-4.60 (1H, m, $CHCH_2S$), 7.42 (1H, t, J 7.5 Hz, Ar-H), 7.56 (1H, d, J 7.5 Hz, Ar-H), 7.90 (1H, d, J 7.5 Hz, Ar-H), 8.01 (1H, s, Ar-H). m/e (fast atom bombardment) 383 (MH^+); Anal. [Found: C, 53.55%; H, 5.82%; N, 7.23; %. Calcd. for $C_{17}H_{22}N_2O_6S$: C, 53.39%; H, 5.80%; N, 7.32%].

Incubation of 9 with IPNS

9 (1.4 mg) Was incubated with IPNS (3.5 I.U) following the published procedure.[6,7,12] Purification of the crude incubation mixture by h.p.l.c. (reverse phase octadecylsilane column, mobile phase = 3% CH_3CN/97% 10mM aqueous NH_4HCO_3) gave 10 (1.0 mg, 72%, calibration against internal dioxan standard).

(2S,5R,6R)-6-(m-carboxyphenylacetyl)-3,3'-dimethyl-7-oxo-1-aza-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (10)

^1H n.m.r. (300 MHz, D_2O) 1.30 (3H, s, CH_3), 1.38 (3H, s, CH_3), 3.55, 3.62 (2H, ABq, J 15 Hz, CH_2Ar), 4.07 (1H, s, 2-H), 5.25, 5.35 (2H, 2 x d, J 4 Hz, 5-H, 6-H), 7.32-7.40 (2H, m, Ar-H), 7.69-7.73 (2H, m, Ar-H); [Calibrated against (2,2,3,3-$^2H_4$)-3-trimethylsilylpropionate (TSP) 0.00 p.p.m.] H.p.l.c. retention time = 5 minutes; m/e (fast atom bombardment) 379 (MH^+).

Determination of Kinetic Parameters

K_m and V_{max} were determined using the integrated form of the Michaelis-Menten equation:

$$V_m t = S_o + S_t + K_m \ln \frac{(S_o)}{(S_t)}$$

where V_m = apparent maximal velocity, t = time, S_o = initial substrate concentration, S_t = substrate concentration at time t, K_m = apparent Michaelis constant. This was transformed into a distribution free plot of K_m/V_m versus $1/V_m$, where the slope of the line = $\frac{-(S_o - S_t)}{\ln \frac{(S_o)}{(S_t)}}$, the ordinate intercept = $t/\ln \frac{(S_o)}{(S_t)}$ and the abscissa intercept = $t/(S_o - S_t)$. Kinetic parameters were determined using a computer program.[14] Parameters for the conversion of ACV (1a) to Isopenicillin N (2a) agreed closely with those previously obtained,[7] using a coupled assay [IPNS-(β-lactamase 1, from Bacillus cereus)] monitoring pH changes.

REFERENCES

1. This work has been reported in a preliminary form: 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.
2. 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.
3. J.E. Baldwin, E.P. Abraham, G.L. Burge, and H-H. Ting, J.Chem.Soc., Chem.Commun., 1985, 1808.
4. J.E. Baldwin, S.R. Herchen, B.L. Johnson, M. Jung, J.J. Usher, and T. Wan, J.Chem.Soc., Perkin Trans. 1, 1981, 2253.
5. T. Tsuji, T. Kataoka, M. Yoshioka, Y. Sendo, and Y. Nishitani, Tetrahedron Lett., 1979, 2793.
6. 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; J.E. Baldwin, J. Gagnon, and H-H. Ting, FEBS Lett., 1985, 188, 253.
7. J.E. Baldwin, S.E. Moroney, and H-H. Ting, Anal.Biochem., 1985, 145, 183.
8. 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; Idem, Tetrahedron, in press.
9. R.D.G. Cooper, Eli Lilly & Co., Indianapolis, U.S.A., personal communication.
10. J.E. Baldwin, R.M. Adlington, R.H. Jones, C.J. Schofield, C. Zaracostas, and C.W. Greengrass, Tetrahedron, 1986, 42, 4879.
11. G. Just and K. Grozinger, Synthesis, 1976, 457.
12. J.E. Baldwin, A.J. Pratt, and M.G. Moloney, Tetrahedron, 1987, 43, 2565.
13. J.E. Baldwin and G. Burge, unpublished results.
14. M.J.C. Crabbe, Comput.Biol.Med., 1985, 15, 111.