Journal of Natural Products
Vol. 52, No. 1, pp. 203-206, Jan-Feb 1989

BIOTRANSFORMATION OF VANCOMYCIN B TO VANCOMYCIN HEXAPEPTIDE BY A SOIL MICROORGANISM

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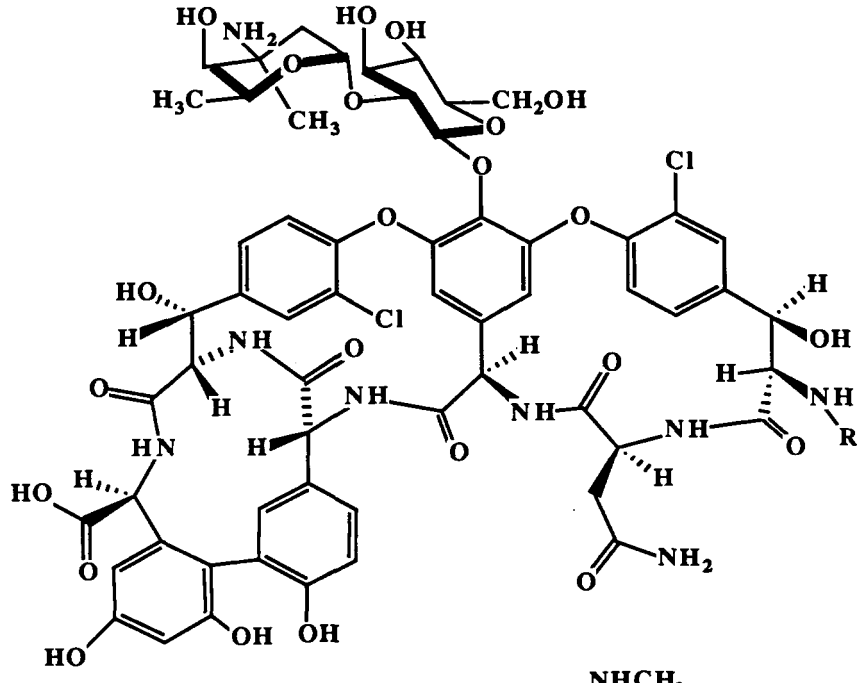
Vancomycin B [**1**] is a glycopeptide antibiotic isolated from *Amycolatopsis orientalis* (1). Many natural and semisynthetic derivatives of these compounds have been reported (2–4), but little information exists on the bioconversion of glycopeptide antibiotics. The only studies to date have been on the aridicins (5) in which protoplasts of the producing culture, *Kibdelosporangium aridum*, have been shown to add sugars to the aglycone of the peptide core. We were interested in modifications of the peptide core of the molecule in such a way as to provide the opportunity of generating a series of new members of this class of compounds.

The mode of action of this class of antibiotics is to interfere with cell wall formation by binding to terminal D-alanyl-D-alanine moieties of the developing bacterial cell wall. Mechanism of action studies have shown that the amide nitrogens at the *N*-terminus of the antibiotic form a "carboxylate binding pocket" (6) that secures the carboxylate group of terminal D-alanyl-D-alanine. Ether coupling of the aromatic groups of the antibiotic serves to maintain the proper "pocket" conformation. Removal of the *N*-methyl-D-leucyl group of vancomycin B might be expected to disrupt the binding pocket enough to destroy antibacterial activity. However, this peptide core molecule would present the opportunity to prepare chemically modified derivatives with restored and perhaps enhanced biological activity.

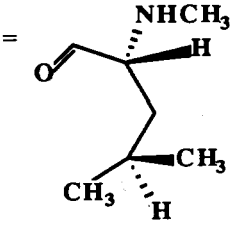
A screen was devised that would augment the possibility of finding an organism to remove the *N*-methyl leucyl group from vancomycin. We employed D-leucyl-D-tyrosine as a carbon source in agar plates seeded with soil suspensions. This dipeptide **2** approximates the *N*-terminal dipeptide of the antibiotic. It was reasoned that an organism that could digest this dipeptide might also have the capability of catalyzing the desired transformation. Isolates that apparently used this dipeptide for growth were selected and tested to determine what effect, if any, they had on the biological activity of vancomycin B. Of the first 13 strains tested, one completely inactivated the antimicrobial activity of vancomycin.

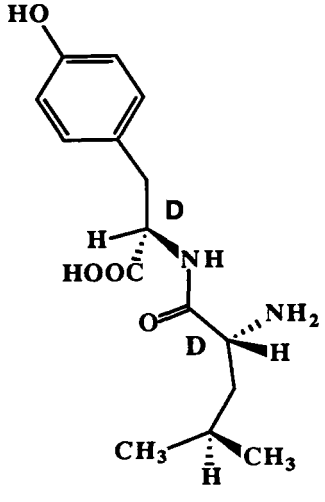
Hplc time course studies of the bioconversion broth from the culture that inactivated vancomycin B demonstrated that as the peak for vancomycin (16 min) disappeared, a new polar peak (11 min) developed in the chromatogram. All of the antimicrobial activity and the peak at 16 min disappeared, and more than 90% of the vancomycin was converted to this new peak based on area under the curve. Using a three-step isolation procedure, material of 94% purity was obtained. The exact mass was correct for a compound that had lost the *N*-terminal leucine, and ^{1}H nmr showed loss of all leucine protons. Chemical reacylation of this material with D-leucine yielded A51568A, *N*-demethylvancomycin (7). The proposed structure for this bioconversion product is vancomycin hexapeptide [**3**].

This report discloses a novel bioconversion of vancomycin to the biologically inactive vancomycin hexapeptide and represents the only bioconversion of a glycopeptide antibiotic carried out by a non-glycopeptide-producing culture. This soil microbe is also the only micro-



1 R=





3 R=H

2

organism reported to inactivate a member of this class of antibiotics. The exact mechanism by which the soil isolate carries out this transformation is unknown; however, preliminary studies indicate the absence of a D-leucine aminopeptidase in this organism (data not shown). The availability of vancomycin hexapeptide as a substrate for a synthetic modification program will allow for a better understanding of the mechanism of action of these compounds. In the future, it also may be important in understanding and overcoming the emerging glycopeptide antibiotic resistance in pathogenic bacteria (8). While this work

was in progress, a report appeared on the preparation of vancomycin hexapeptide using a modified Edman degradation (9).

EXPERIMENTAL

SCREENING METHOD.—A sample (0.1 g) of soil was suspended in 10 ml of sterile distilled water and diluted 1/1000. This suspension (100 µl) was plated on DLDT agar medium consisting of D-leucyl-D-tyrosine (Research Plus) (2.0 g), $(NH_4)_2SO_4$ (2.0 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), Noble agar (12 g), and 1 liter distilled H_2O, adjusted to pH 7.0. Colonies that grew on this medium were then streaked for isolation on another DLDT agar plate to insure that the colony was pure. These cultures were maintained on Bennett's agar.

BIOCONVERSION OF VANCOMYCIN.—Individual soil isolates were inoculated into TSBD medium consisting of T-soy broth base (30 g), potato dextrin (20 g), and 1 liter of distilled H_2O. The cultures were allowed to grow for 2 days at 30° with shaking (250 rpm). These vegetative cultures were used to inoculate CSI medium consisting of Nutrisoy flour (15 g), casein (1.0 g), dextrose (25 g), blackstrap molasses (3.0 g), $CaCO_3$ (2.5 g), Czapeck's mineral solution (2.0 ml), and 1 liter of distilled H_2O, adjusted to pH 7.4. After 2 to 4 days growth at 30° and 250 rpm, vancomycin B (200 µg/ml, final concentration) was added to the cultures, which were incubated for an additional 24 to 48 h. After this time, the pH of the broth was adjusted to 10.5 and the cells removed by centrifugation. The supernatant was decanted and the pH returned to 7.0. Samples of the broth were padded out against a susceptible test organism to determine the biological activity remaining. The organism that was used in this study was later identified as *Actinomadura citrea* (NRRI 18382).

ISOLATION OF VANCOMYCIN HEXAPEPTIDE.—Bioconversion fermentation broth (7 liters) to which 2.0 g of vancomycin B had been added was filtered using 3% Hyflo Supercel. The mycelial mat was discarded and the pH of the filtrate was adjusted to 7.0 with 1.0 N HCl. Hplc analysis of the broth filtrate indicated that approximately 90% of the original vancomycin B was converted to vancomycin hexapeptide. The filtrate was applied to a column of Diaion HP-20 resin (6.0 × 53 cm) equilibrated in H_2O. The column was washed with 4.0 liters of H_2O and eluted with three 850-ml portions of 50% MeOH in H_2O. The eluate was concentrated to a residue, redissolved in 750 ml of H_2O, and lyophilized, yielding 26 g of material. Hplc analysis showed the sample to be 5.3% pure thus representing 1.4 g of vancomycin hexapeptide.

Crude vancomycin hexapeptide (1.5 g, 5.3% purity) was dissolved in 20 ml of H_2O and applied to a column of Sephadex CM-25 (2.8 × 50 cm; NH_4^+ cycle, equilibrated with H_2O). The column was initially washed with 60 ml of H_2O and then eluted with a linear gradient using 500 ml each of H_2O and 0.25 M $(NH_4)HCO_3$. The fractions containing the compound of interest were pooled and applied to a column of Diaion HP-20 resin (1.2 × 30 cm, equilibrated in H_2O). After loading, the column was washed with 100 ml of H_2O to remove inorganic salts. The desired material was eluted from the column in a single 50-ml portion of 50% MeOH in H_2O (flow rate 2 ml/min). The HP-20 eluate was concentrated to a residue and redissolved in 15 ml of H_2O. It was necessary to lower the pH to effect solution. Lyophilization yielded 23 mg of vancomycin hexapeptide (94% pure).

HPLC SYSTEM FOR SEPARATION OF VANCOMYCIN AND VANCOMYCINHEXAPEPTIDE.—Hplc was performed using a C_{18} (IBM Octadecyl, 4.5 × 150 mm, 5 µ) column and a gradient system consisting of MeOH (solvent A) and 0.05 M ammonium trifluoroacetate at pH 3.0 (solvent B). The gradient started with 5% solvent A and 95% solvent B. From 1 to 11 min, the percent of A rose to 35 and was held there until the end of the run (19 min). The flow rate was 1 ml/min and the uv detector was set at 240 nm.

STRUCTURE ELUCIDATION OF VANCOMYCIN HEXAPEPTIDE.—Uv spectrophotometric analysis gave a λ max of 281 nm (e = 5780) under neutral conditions (EtOH). Fabms gave a P + 1 of 1321 (integer mol wt is 1320). Calculated exact mass for $C_{59}H_{63}N_8O_{23}Cl_2$ is theory, 1321.3383 and found, 1321.3374. First order interpretation of the 300 MHz ^1H nmr demonstrated the absence of the alpha (3.70 ppm), beta (1.65, 1.5 ppm) and *gem*-dimethyl protons (0.9 ppm) of *N*-methylleucine.

REACYLATION AND DEBLOCKING OF VANCOMYCIN HEXAPEPTIDE.—A total of 100 mg (0.075 mmol) of vancomycin hexapeptide was dissolved in 5 ml of anhydrous DMF, followed by the addition of 50 mg (0.14 mmol) of *N-t*-Boc-D-leucyl-*O*-benzotriazole. After 20 min at ambient temperature under N_2, tlc indicated that the reaction was complete. Removal of solvent and trituration with Et_2O-CH_2Cl_2 (1:1) followed by filtration yielded 118 mg of a tan glass. Fabms of the compound gave P + 1 of 1534 (integer mol wt 1533, empirical formula $C_{70}H_{81}N_9O_{26}Cl_2$). ^1H-nmr showed that the leucine protons were reintroduced. The compound was then purified on a micro-preparative column (Waters C_{18} µBondapak, 19 × 50 mm) using 25% MeCN/H_2O containing 1% $(NH_4)H_2PO_4$. After desalting over HP-20ss (Diaion), 22 mg of the *N-t*-Boc A51568A (*N-t*-Boc *N*-demethylvancomycin) was

obtained. Trifluoroacetic acid (3 ml) was added to 20 mg of the compound at $-10°$ with stirring. After 15 min at $-10°$, the solvent was removed and the resulting syrup triturated with CH_2Cl_2 (3×10 ml). The precipitate was redissolved in H_2O, pH adjusted to 6.2 with pyridine, and the solution lyophilized yielding 12 mg of a tan solid. Fabms of a sample of the compound gave P + 1 of 1434. A diagnostic peak at 1128 was also found. This corresponds to the aglycone of A51568A which confirmed that the acylation occurred at the *N* terminus of the peptide core and not the amino-sugar nitrogen. Exact mass for $C_{65}H_{74}N_9O_{24}Cl_2$ is theory, 1434.4224 and found, 1434.4191. Amino acid analysis showed the presence of one leucine for every aspartate in the molecule. Bioautography on tlc gave a single bioactive spot with the same R_f(0.4) as A51568A (7) against *Micrococcus luteus*.

ACKNOWLEDGMENTS

The authors thank Mr. L. Boeck's laboratory for fermentations of the soil isolate, Mr. P. Baker's laboratory for running the initial step in our isolation scheme, and Mr. J. Occolowitz for mass spectral analysis of vancomycin hexapeptide.

LITERATURE CITED

1. M.H. McCormick, W.M. Stark, G.E. Pittenger, R.C. Pittenger, and J.M. McGuire, in: "Antibiotics Annual 1955–1956". Ed. by H. Welch and F. Marti-Ibañez, Medical Encyclopedia, New York, 1956, p. 606.
2. M.C. Shearer, P. Actor, B.A. Bowie, S.F. Grappel, C.H. Nash, D.J. Newman, Y.K. Oh, C.H. Pan, and C.J. Nisbet, *J. Antibiot.*, **38**, 55 (1985).
3. L.D. Boeck, F.P. Mertz, and G.M. Clem, *J. Antibiot.*, **38**, 1 (1985).
4. C.M. Harris, S.W. Fesik, A.M. Thomas, R. Kannan, and T.M. Harris, *J. Org. Chem.*, **51**, 1509 (1986).
5. S.K. Chung, Y.K. Oh, P. Taylor, R. Gerber, and L.J. Nisbet, *J. Antibiot.*, **39**, 652 (1986).
6. D.H. Williams and J.P. Waltho, *Biochem. Pharmacol.*, **37**, 133 (1988).
7. A.H. Hunt, G.G. Marconi, T.K. Elzey, and M.M. Hoehn, *J. Antibiot.*, **37**, 917 (1984).
8. P.S. Schwabe, J.T. Stapleton, and P.H. Gilligan, *N. Engl. J. Med.*, **316**, 927 (1987).
9. P.M. Booth, D.J.M. Stone, and D.H. Williams, *J. Chem. Soc., Chem. Commun.*, 1694 (1987).

Received 3 August 1988