MURACEINS — MURAMYL PEPTIDES PRODUCED BY *NOCARDIA ORIENTALIS* AS ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

KAREN BUSH, PAUL R. HENRY and DOROTHY S. SLUSARCHYK

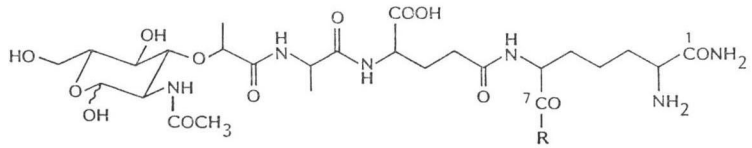
The Squibb Institute for Medical Research
P.O. Box 4000, Princeton, New Jersey 08540, U.S.A.

(Received for publication January 23, 1984)

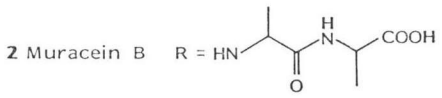
Three muraceins, a family of muramyl peptides, were isolated from *Nocardia orientalis* as inhibitors of angiotensin-converting enzyme (ACE). Muracein A, the most potent inhibitor, inhibited ACE competitively with a *Ki* of 1.5 μM. Liver alcohol dehydrogenase and carboxypeptidase A, two other zinc-containing enzymes, were not inhibited at 150 μM. Inhibition of ACE could not be reversed by divalent cations.

Angiotensin-converting enzyme (ACE) is recognized as a pharmacologically important target in the treatment of hypertension. Clinically-effective ACE inhibitors include the peptide analog captopril[1] and teprotide, a nonapeptide isolated from the venom of *Bothrops jararaca*[2]. Recently two novel ACE inhibitors have been isolated from *Streptomyces* strains: ancovenin[3], an unusual peptide containing sixteen amino acids, and L-681,176, a small tricarboxylic acid peptide analog[4].

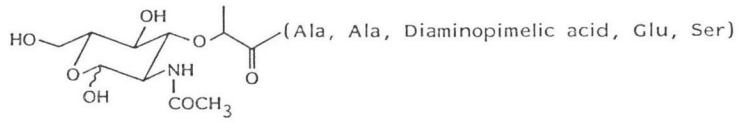
Fig. 1. Structures of muraceins A, B, C and SQ 28,370.



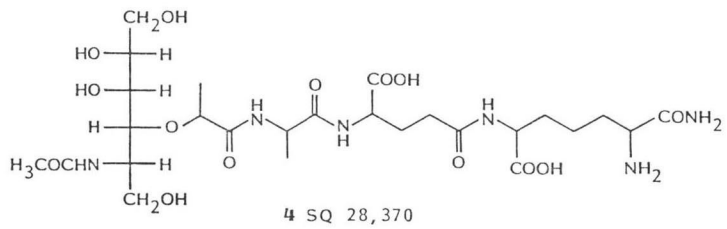
1 Muracein A R = OH



2 Muracein B R = HN



3 Muracein C



4 SQ 28,370

During the course of screening for naturally-occurring ACE inhibitors, we identified a family of novel muramyl peptides from *Nocardia orientalis*. These molecules which have been named muraceins* (Fig. 1), inhibit ACE over a wide range of concentrations. Taxonomy of the producing organism, fermentation and biological properties of the muraceins are discussed in this paper. Isolation and structure determination are described in the accompanying paper[5].

Taxonomy

Culture SC 12,842 was isolated from a soil sample collected in Hamilton Township, New Jersey. SC 12,842 was identified as a strain of *Nocardia orientalis* based on the following information: The organism is non-acid fast and produces a characteristic fragmenting vegetative mycelium and rudimentary aerial mycelium. Cell wall acid hydrolysates prepared by the method of BECKER *et al.*[6] and analyzed by thin-layer chromatography[7] contained *meso*-diaminopimelic acid, with galactose and arabinose as the major sugar components. This type IV cell wall is typical of the nocardiae[8].

Comparison of SC 12,842 with *N. orientalis* ATCC 19795 is presented in Table 1. These results concur with those of GORDON *et al.*[9]. Therefore, SC 12,842 and *N. orientalis* are considered to be identical. The culture has been deposited in the American Type Culture Collection, where it has been assigned the number ATCC 39444.

During our screening program, muracein production was identified in three other *Nocardia* strains isolated from soil samples. Ten known *Nocardia* species were found to be nonproducers of muraceins.

Table 1. Comparison of SC 12,842 with *Nocardia orientalis* ATCC 19795.

Characters	SC 12,842	*N. orientalis*
Morphology:		
Fragmenting mycelium	+	+
Cell wall:		
meso-DAP	+	+
Galactose, arabinose	+	+
Decomposition of:		
Adenine	−	−
Casein	+	+
Testosterone	+	+
Tyrosine	+	+
Xanthine	±	−
Resistance to:		
Lysozyme	−	−
Rifampin	−	−
Acid from:		
Adonitol	+	+
Cellobiose	+	+
meso-Erythritol	+	+
Lactose	+	+
Maltose	+	+
Melezitose	−	−
α-Methyl-D-glucoside	+	+
Urease	+	+

Materials and Methods

Preparation of ACE

ACE was prepared by suspending 10 g of rabbit lung acetone powder (Pel-Freez Biologicals) overnight in 120 ml of 50 mM phosphate buffer, pH 8.3 at 4°C. The suspension was homogenized in a blender for 3 minutes and centrifuged to remove solids (40,000 *g* for 1 hour at 4°C). The supernatant (110 ml) was then eluted from Sephadex G-75 (8×60 cm) with 50 mM Tris-HCl, pH 7.5. Column fractions were assayed for ACE hydrolytic activity. Active fractions were pooled and used as the enzyme source for screening and kinetic studies.

Assays for ACE Activity

Assays were performed spectrophotometrically by following hydrolysis of either furanacryloylphenylalanylglycylglycine (FAPGG)[10] at 25°C or *p*-nitrobenzyloxycarbonylglycyl(*S*-4-nitrobenzo-2-oxa-1,3-diazole)-L-cysteinylglycine (NBGCG)[11] at 37°C in 50 mM Tris-HCl buffer containing 0.3 M

* The muracein nomenclature is derived from the words *MUR*amyl peptide *ACE IN*hibitor.

NaCl, pH 7.5. Hydrolysis of FAPGG was monitored at 345 nm; NBGCG hydrolysis was followed at 475 nm.

Gilford spectrophotometers, either Model 250 or Model 203S, were used to measure hydrolysis rates for kinetic studies and I_{50} determinations. *Ki* determinations were calculated using linear regression analyses of data plotted according to LINEWEAVER-BURK and DIXON[12].

Screening of fermentation broth supernatants was performed utilizing a Technicon Auto Analyzer II system. In this procedure, one volume of ACE was mixed with an equal volume of inhibitor (supernatant) before two volumes of 80 μM NBGCG was added. A reaction time of about 40 minutes was employed before final absorbances were recorded. Captopril at 1.0 nM was used as a positive control throughout the assay.

Effects of ACE inhibitors on smooth-muscle contraction were tested in excised guinea pig ileum using the method of RUBIN and collaborators[13,14].

Fermentation

Seed cultures of *N. orientalis* SC 12,842 were prepared by transferring a loopful of surface growth from an agar slant into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: 0.40% yeast extract, 1.0% malt extract and 0.40% dextrose in distilled water. The pH was adjusted to 7.3 before sterilization. The flasks were then incubated at 25°C on a rotary shaker (300 rpm, 5-cm stroke) for approximately 72 hours.

A 3% (v/v) transfer was made from the seed culture flasks to 500-ml Erlenmeyer flasks containing 100 ml each of the following: $(NH_4)_2SO_4$ 0.264%, K_2HPO_4 0.43%, KH_2PO_4 0.238%, $MgSO_4 \cdot 7H_2O$ 0.10% and trace salt solution 0.10% (v/v) in distilled water. The salt solution contained $CuSO_4 \cdot 5H_2O$ 0.64%, $FeSO_4 \cdot 7H_2O$ 0.11%, $MnCl_2 \cdot 4H_2O$ 0.79% and $ZnSO_4 \cdot 7H_2O$ 0.15% in distilled water. The pH was adjusted to 6.8~7.0 before sterilization. At the time of inoculation 5.0 ml of sterile glucose (20%) was added to each flask. The flasks were then incubated at 25°C on a rotary shaker (300 rpm, 5-cm stroke) for approximately 120 hours. Inhibition of ACE activity was monitored using NBGCG as substrate in the auto analyzer assay. Occasional fermentations were allowed to proceed for 144 hours for maximum production of the muraceins.

At harvest the flask contents were pooled. After centrifugation, the resulting supernatant was used for the isolation of muraceins.

Biological Activity

ACE Inhibition

Muracein A and SQ 28,370 (reduction product of muracein A) inhibited ACE with I_{50} values comparable to that observed for the snake venom peptide, teprotide (Table 2). Muraceins B and C were considerably less potent inhibitors. Inhibition of ACE was not time-dependent when studied from 1 to 60 minutes. However, I_{50} values for the muraceins were lowered 20~30% if enzyme and inhibitor were preincubated briefly (less than one minute) before substrate was added to the reaction. This preincubation effect may reflect a slow equilibration of the enzyme-inhibitor complex.

Muracein A behaved as a pure competitive inhibitor of ACE with a *Ki* value of 1.5 μM, as shown in Fig. 2. A strong specificity for inhibition of ACE was exhibited by muracein A; at 150 μM less than 5% inhibition was observed with liver alcohol dehydrogenase and carboxypeptidase A, two other zinc-containing enzymes. Although a ligand may be formed between the zinc ion of ACE and carbonyl or carboxylate groups of the muraceins, strong chelation of divalent cations was not a characteristic of these inhibitors. This conclusion was also supported by the observation that inhibition of ACE by muracein A was not affected by the presence of 1.0 mM $ZnCl_2$, $CaCl_2$ or $CoCl_2$.

When muracein A was tested for its effect on smooth-muscle contracting activity in the excised guinea pig ileum, the EC_{50}* for AI antagonism was 64 μM; the EC_{50} for bradykinin potentiation was

* Effective concentration producing a 50% change in agonist effect.

Fig. 2. LINEWEAVER-BURK plot of inhibition of ACE by muracein A.

ACE and inhibitor or buffer were incubated 5.0 minutes in a volume of 65 μl at 25°C before addition of 1.0 ml FAPGG (prewarmed to 25°C). Muracein A concentrations: ○, 0 μM; △, 2.0 μM; □, 5.1 μM.

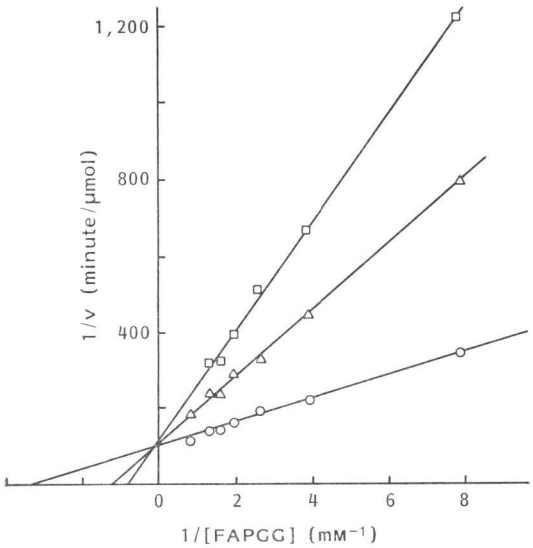


Table 2. I_{50} Values of selected ACE inhibitors.

Rabbit lung ACE (200 μl) and inhibitor or buffer (50 μl) were incubated 45 seconds at 20°C before 0.20 mM NBGCG (800 μl) was added to initiate reaction. Hydrolysis rates were monitored at 37°C.

Inhibitor	I_{50} (nM)
Captopril[a]	0.65
Teprotide[b]	130
Muracein A	280
SQ 28,370	120
Muracein B	12,000
Muracein C	170,000

[a] D-3-Mercapto-2-methylpropenoyl-L-proline
[b] <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro

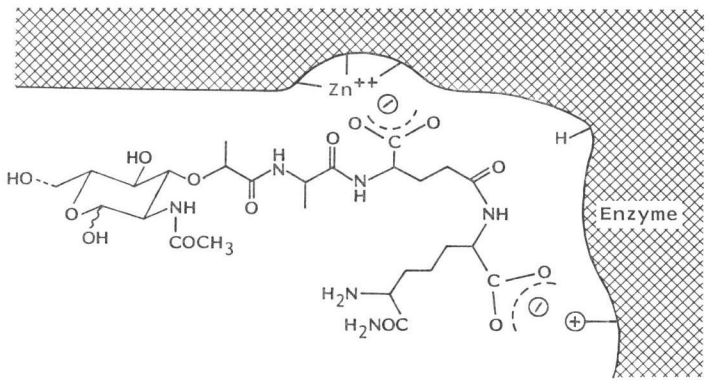
3.9 μM. The activity profile generated from these results supported the previous observations that muracein A was a specific ACE inhibitor.

Muracein A exhibited no antifungal or antibacterial activity when tested against a variety of *Candida* sp., Gram-negative and Gram-positive microorganisms.

Discussion

The observed selectivity for ACE inhibition must involve specific interactions of muraceins with the enzyme active site. One representation of muracein binding to ACE is shown in Fig. 3. For all the muraceins one can envision a ligand forming between the active site zinc and the carboxylate or carbonyl functionalities shown; however, this by itself could not account for the observed differential inhibition of ACE by the various muraceins. The C_7 carboxylate of muracein A must be a critical functionality and may bind at the same site as the terminal carboxyl group of natural ACE substrates. Addition of alanylalanine at this position to give muracein B significantly increased the I_{50} value, suggesting a possible steric interference with binding. Because muracein A and SQ 28,370 inhibit ACE

Fig. 3. Possible interaction of muracein A with the active site of ACE.



with comparable I_{50} values, the muramic acid moiety must not play a major role in determining affinity.

Taxonomic identification of *Nocardia* sp. is based in part upon the presence of diaminopimelic acid in cell wall hydrolysates. Thus, it is quite probable that the muraceins are fragments of *Nocardia* cell wall.

Other muramyl peptides have been described in the literature as molecules with a variety of biological activities. A muramyl peptide isolated from human urine was reported to be a sleep-promoting factor[15]. Muramyl peptides have also been noted for their immunomodulation activities, either as immunosuppressants or as immunostimulants[16]. For example, a liposome-encapsulated muramyl dipeptide analog was recently shown to protect mice against *Candida albicans* infection[17]. However, the ACE inhibitory effects of muramyl peptides have not previously been reported. Therefore, this specific inhibitory activity singles out the muraceins as novel biologically active muramyl peptides.

Acknowledgments

We thank Mr. W. H. TREJO for providing the taxonomic classification for *Nocardia orientalis*, Dr. B. RUBIN and Ms. D. KOTLER for performing the guinea pig ileum assay, Dr. S. K. TANAKA for studying antimicrobial activities and Dr. R. B. SYKES for his generous support.

References

1) ONDETTI, M. A.; B. RUBIN & D. W. CUSHMAN: Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. Science 196: 441~444, 1977
2) FERREIRA, S. H.: A bradykinin-potentiating factor (BPF) present in the venom of *Bothrops jararaca*. Brit. J. Pharmacol. 24: 163~169, 1965
3) KIDO, Y.; T. HAMAKADO, T. YOSHIDA, M. ANNO, Y. MOTOKI, T. WAKAMIYA & T. SHIBA: Isolation and characterization of ancovenin, a new inhibitor of angiotensin I converting enzyme, produced by actinomycetes. J. Antibiotics 36: 1295~1299, 1983
4) HUANG, L.; G. ROWIN, J. DUNN, R. SYKES, R. DOBNA, B. A. MAYLES, D. M. GROSS, J. LIESCH, O. D. HENSENS & R. W. BURG: Discovery of an angiotensin converting enzyme inhibitor, L-681,176 in the culture filtrate of *Streptomyces* sp. 83rd Annual Meeting, Am. Soc. for Microbiol., Abstract O31, New Orleans, 1983
5) SINGH, P. D. & J. H. JOHNSON: Muraceins — Muramyl peptides produced by *Nocardia orientalis* as angiotensin-converting enzyme inhibitors. II. Isolation and structure determination. J. Antibiotics 37: 336~343, 1984
6) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
7) STANECK, J. L. & G. D. ROBERTS: Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. 28: 226~231, 1974
8) LECHEVALIER, H. A.; M. P. LECHEVALIER & N. N. GERBER: Chemical composition as a criterion in the classification of actinomycetes. Adv. Appl. Microbiol. 14: 47~72, 1971
9) GORDON, R. E.; S. K. MISHRA & D. A. BARNETT: Some bits and pieces of the genus *Nocardia*: *N. carnea*, *N. vaccinii*, *N. transvalensis*, *N. orientalis* and *N. aerocolonigenes*. J. Gen. Microbiol. 109: 69~78, 1978
10) HOLMQUIST, B.; P. BÜNNING & J. F. RIORDAN: A continuous spectrophotometric assay for angiotensin converting enzyme. Anal. Biochem. 95: 540~548, 1979
11) PERSSON, A. V.; S. F. RUSSO & I. B. WILSON: A new chromogenic substrate for angiotensin-converting enzyme. Anal. Biochem. 91: 674~683, 1978
12) DIXON, M.: The graphical determination of *Km* and *Ki*. Biochem. J. 129: 197~202, 1972
13) RUBIN, B.; E. H. O'KEEFE, D. G. KOTLER, D. A. DEMAIO & D. W. CUSHMAN: Use of excised guinea pig ileum as a predictive test for inhibitors *in vivo* of angiotensin-converting enzyme (ACE). Fed. Proc. 34: 770, 1975
14) RUBIN, B.; R. J. LAFFAN, D. G. KOTLER, E. H. O'KEEFE, D. A. DEMAIO & M. E. GOLDBERG: SQ 14,225 (D-3-mercapto-2-methylpropenoyl-L-proline), a novel orally active inhibitor of angiotensin I-converting enzyme. J. Pharmacol. Exp. Ther. 204: 271~280, 1978

15) KRUEGER, J. M.; J. R. PAPPENHEIMER & M. L. KARNOVSKY: The composition of sleep-promoting factor isolated from human urine. J. Biol. Chem. 257: 1664~1669, 1982
16) LEDERER, E.: Synthetic immunostimulants derived from the bacterial cell wall. J. Med. Chem. 23: 819~825, 1980
17) FRASER-SMITH, E. B.; D. A. EPSTEIN, M. A. LARSEN & T. R. MATTHEWS: Protective effect of a muramyl dipeptide analog encapsulated in or mixed with liposomes against *Candida albicans* infection. Infec. Immun. 39: 172~178, 1983