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Antimicrobial activity of *o*-carboranylalanine

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Summary. Functionalized polyhedral carboranes, including amino acid analogs, have unique physicochemical properties and are used as experimental anticancer agents. However, our current knowledge on their effect in nonmammalian biological systems is limited. We investigated the activity spectrum *in vitro* of *o*-carboranylalanine (*o*-Cba), considered to be a highly lipophilic analog of phenylalanine, against representative plant pathogenic bacteria and fungi of various taxonomic position. The antibacterial effect of *o*-Cba against some species was comparable to that of the widely used agricultural antibiotic, streptomycin. The sensitivity of individual bacterial species to *o*-Cba within the same genus varied to a greater extent than the average sensitivity of various genera. In general, this carborane-containing amino acid was more toxic to Gram positive bacteria (*Bacillus*, *Corynebacterium*, *Curtobacterium*, *Micrococcus*, *Rhodococcus*, and *Staphylococcus*) than to Gram negative ones (*Agrobacterium*, *Erwinia*, *Escherichia*, *Pseudomonas*, *Rhizobium*, and *Xanthomonas*). Compared to the commercial fungicide, prochloraz, *o*-Cba was weakly toxic against various fungi (Zygo- and Ascomycota). It was also inferior to the commercial fungicide metalaxyl in inhibiting the vegetative growth of oomyceteous plant pathogens (*Pythium irregulare*, *Phytophthora cryptogea* and *Plasmopara halstedii*). Against the asexual spores of *P. halstedii*, *o*-Cba, however, was over a thousandfold more active than tridemorph, a selective zoospore inhibitor fungicide. For all taxonomic groups, the observed antimicrobial effect of *o*-Cba could be diminished with histidine, but not with phenylalanine. In studies on healthy and mildew-infected sunflower and tobacco plants *o*-Cba showed neither fungicidal nor phytotoxic effects at 500 ppm. This is the first report on the biological activity spectrum of a carborane-containing amino acid.

Keywords: Amino acids – *o*-Carboranylalanine – Histidine – Bacteria – Fungi – Oomycota – *Plasmopara*

Introduction

Due to their unique physicochemical properties, organoboron substances with polyhedral cages have attracted much attention (Grimes, 1970). The readily available carboranes, icosahedral systems with two carbon and ten boron atoms, and their substituted derivatives have recently been studied thoroughly as potential tools in boron neutron capture therapy of cancer (Barth et al., 1990; Hawthorne, 1993; Soloway et al., 1998). Among the broad range of carborane-derivatives prepared as latent energy-containing "Trojan horses" for target-selective delivery, amino acid analogues offer especially promising means since these compounds can be readily incorporated into carrier peptides. Based on the near sterical equivalence of the rotating phenyl ring and the spherical carborane cage, isomers of carboranylalanine have been the amino acids of choice in this respect. Racemic *o*-carboranylalanine (*o*-Cba), or (*RS*)-3-(1,2-dicarba-*closo*-dodecaborane-1-yl)-2-aminopropanoic acid (Fig. 1), was first prepared independently by Brattsev and Stanko (1969) and Zakharkin et al. (1970). L-*o*-Cba with the stereochemistry corresponding to that of L-Phe was first synthesized by Leukart et al. (1976) from L-propargylglycine and was used to replace L-Phe in [Leu]-enkephalin (Eberle et al., 1977), in tripeptide inhibitors of chymotrypsin (Fischli et al., 1977), in a bradykinin analogue (Couture et al., 1979) and in angiotensin II (Escher et al., 1980). Recently, enantioselective synthesis of L-*o*-Cba has also been described (Karnbrock et al., 1995; Radel and Kahl, 1996). Radel and Kahl (1996) prepared the D-enantiomer as well. The related (*S*)-2-Me-*o*-Cba and (*S*)-*p*-Cba have also been synthesized (Malmquist and Sjöberg, 1996).

The chemistry of the various derivatives of carboranylalanine has been the subject of extensive studies but systematic biological studies have been lacking. Recently Yong et al. (1995) found that solubilized racemic *o*-Cba was cytotoxic against human proliferating melanoma cells *in vitro* with an unknown mode of action. Simple mono-, di- and tetracage carboranes, including *o*-carborane, 2-methyl-1-carboranecarboxylic acid and a series of nitrogen- and sulphur-containing derivatives, exhibited high selective activity against certain fungi and bacteria (Totani et al., 1981).

The growing interest in the chemical and clinical uses of carboranes, amino acid derivatives and peptides in particular, necessitates a detailed examination of their effects on various biological systems. In connection with our research on carborane-containing substances with potential application in agriculture (Nachman et al., 1996), we investigated the biological activity spectrum of *o*-Cba on 44 microbial species representing various taxa. Most of the bacteria and fungi tested are of agricultural importance. For two obligate parasites, the effect of *o*-Cba on their host plants was also evaluated.

Materials and methods

Synthesis and characterization

Racemic *o*-Cba of min. 95% purity was synthesized as described recently (Wyzlic et al., 1996) except we used liquid-liquid phase transfer catalytic alkylation (O'Donnel and

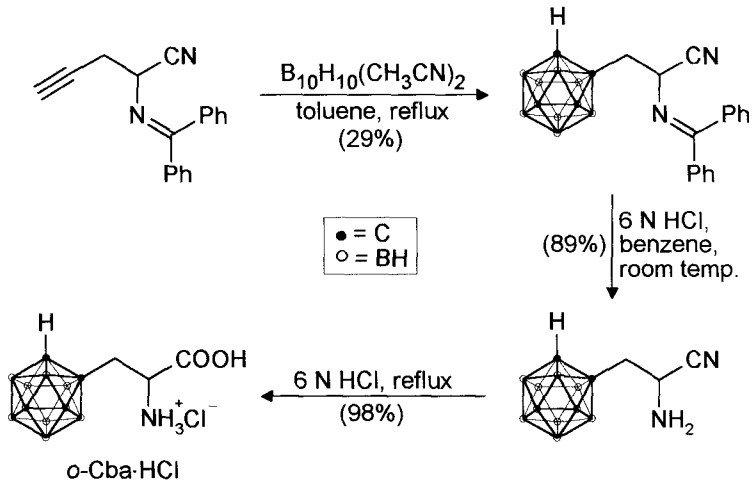

Fig. 1. Synthesis of racemic *o*-carboranylalanine (*o*-Cba)

Eckrich, 1978) for the preparation of the diphenylmethylene-protected glycine derivative starting material (Fig. 1).

The spectral properties of the hydrochloride salt of the title product were in agreement with those reported earlier (Karnbrock et al., 1995; Wyzlic et al., 1996). ^{1}H-NMR (CD_3OD): δ 1.6–3.0 (br, 10H, BH), 2.70 (dd, J = 6.0 and 16.0 Hz, 1H of CH_2), 3.13 (dd, J = 6.0 and 16.0 Hz, 1H of CH_2), 4.10 (t, J = 6.0 Hz, 1H, CH), 4.65 (s, 1H, CH in carborane); ^{13}C-NMR (CD_3OD): δ 38.4, 53.7, 61.8, 72.3, 170.1; IR (KBr) ν 3,420, 3,057, 2,942, 2,591, 1,750, 1,490 cm^{-1}; FAB-MS: m/z $[M+H]^+$ = 232 (within the boron cluster envelope); calculated: 232.3 for $C_5H_{18}B_{10}NO_2$.

Due to its low aqueous solubility, 0.2 M *o*-Cba HCl stock solution was prepared in a mixture of DMSO and methyl alcohol (1:3). Analytical grade mineral salts, glucose, glycerol and amino acids (REANAL, Budapest), and vitamins (CHINOIN, Budapest), and bacteriological agar No. 1 (OXOID, Basingstoke) were used. Streptomycin sesquisulphate was the gift of BIOGAL (Debrecen). Metalaxyl, tridemorph, and prochloraz were isolated from the commercial preparations Ridomil (Ciba Geigy, Basel), Calixin 75EC (BASF, Ludwigshafen), and Sportak (Schering, Berlin), respectively.

Test organisms

Plants: Leaves of tobacco (*Nicotiana tabacum* L. cv Samsun) and germlings of sunflower (*Helianthus annuus* L. cv GK-70) were used for assessing phytotoxicity.

Oomycota: two facultative parasites, *Pythium irregulare* (Buism.) and *Phytophthora cryptogea* (Pethyb et Laff.) were maintained on green pea agar (GPA). Mycelial discs cut from the edge of four day old colonies grown on GPA were used as inocula. The obligate parasite, *Plasmopara halstedii* (Farl.) Berlese et de Toni race 1, was maintained on sunflower plants as described earlier (Oros and Virányi, 1987).

Fungi: The following species of Zygo- and Ascomycota were tested: *Alternaria mali* Rob., *Aspergillus niger* van Tiegh., *Botrytis allii* Munn., *B. cinerea* Pers., *Cladosporium cucumerinum* Ellis et Arth., *Erysiphe cichoracearum* D.C., *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyder et Hansen, *Glomerella cingulata* (Ston.) Spaudl. et Schrenk., *Mucor racemosus* L., *Mycosphaerella tulasnei* (Janz.) Land., *Myrothecium roridum* Tode, *Penicillium oxalicum* Currie et Thom., *Rhizopus nigricans* Ehrenberg, *Thielaviopsis basicola* (Berk. et Broome) Ferraris, *Trichoderma harzianum* Rifai, *Trichotecium roseum* Link. Saprophytic and facultative parasitic fungi were maintained on Potato-Dextrose

Agar slants. For producing conidia these strains were grown on Malt Agar slants containing inorganic salts ($NaNO_3$, KCl, KH_2PO_4, K_2HPO_4 and $MgSO_4$ at 3.0, 0.5, 0.5, 0.5 and 0.5 g/l, respectively) and vitamins (pyridoxine HCl, thiamin HCl, riboflavin and nicotinamide at 1.0, 10.0, 1.0 and 20.0 mg/l, respectively) and citric acid (0.2 g/l). The obligate parasite *E. cichoracearum*, the causative agent of the powdery mildew disease, was maintained on greenhouse grown tobacco plants. Conidia of test fungi were suspended in sterile distilled water (10^5 cell/ml) containing glucose and TWEEN 40 (2 and 0.2 g/l, respectively).

Bacteria: The following species were tested: *Agrobacterium radiobacter* (Beijerinck et vanDelden) Conn, *A. tumefaciens* (Smith & Townsend) Conn, *Bacillus subtilis* Cohn, *B. thuringiensis* Berliner subsp. *kurstaki*, *Corynebacterium michiganense* (E. F. Smith) Jensen, *Curtobacterium flaccumfaciens* (Hedges) Collins et Jones, *Escherichia coli* (Migula) Castellani et Chalmers, *Erwinia atroseptica* Dye, *E. herbicola* (Löhnis) Dye, *E. uredovora* Dye, *Micrococcus luteus* (Schroeter) Cohn, *Pseudomonas fluorescens* Migula, *P. phaseolicola* (Burkholder) Dowson, *P. syringae* van Hall, *Rhizobium trifolii* Dangeard, *Rhodococcus fascians* (Tilford) Goodfellow, *Staphylococcus aureus* Rosenbach, *Xanthomonas alfalfae* (Riker, Jones et Davis) Dowson, *X. malvacearum* (Erw. Smith) Dowson, *X. phaseoli* Burkholder, *X. stewartii* (Erw. Smith) Dowson. The bacteria were maintained on Nutrient Agar (Oxoid CM3) completed with vitamins (nicotinamide, pyridoxine HCl, riboflavin and thiamin HCl at 20.0, 1.0, 1.0, and 10.0 mg/l, respectively). Standard suspensions were prepared by washing cells from slants of 24 h old cultures grown at 21(±1)°C. The cells were centrifuged, then suspended in sterile distilled water (5×10^7 cell/ml) and these suspensions were used as inoculum.

All test organisms were from the collection of Plant Protection Institute, HAS.

Determination of biological activities

Ten and two-fold dilution series were prepared either in sterile distilled water or in appropriate nutrient medium where the drug concentration ranged from 10^{-10} M to 5×10^{-3} M. The minimum inhibitory concentration (MIC) and maximum tolerated concentration (MTC) values for each case were determined.

Phytotoxicity. Method A: The aqueous solution of *o*-Cba at appropriate concentrations was infiltrated into the leaf sheets of tobacco plant and evaluations were done after 2, 24, 48 and 72 h by comparing the treated area to control areas infiltrated with distilled water (Oros et al., 1989). Method B: Sunflower germlings were immersed into the aqueous solution of *o*-Cba at appropriate concentrations for 18 h then sowed into the soil/sand mixture (3:1 by weight) in 0.5 kg pots. Their development was followed to four-leaf stage and plants treated with *o*-Cba and distilled water (control) were compared for any morphological alteration.

Antioomycete activity. The effect on the growth of the vegetative thallus of both *Pythium* and *Phytophthora* was tested in axenial cultures on GPA according to Oros and Kőmíves (1991). The antiperonospora activity was examined on sunflower germlings *post infectionem* according to Oros and Virányi (1987). To assess the effect of *o*-Cba on asexual spores of *Plasmopara*, the following parameters were recorded: viability of zoosporangia, germination of zoosporangia, viability of zoospores (motion and plasmalemma semipermeability) and cystospore germination. All these events were observed microscopically in suspensions of spores (2×10^5 cell/ml) mixed (1:1 by volume) with solutions of compound at appropriate concentrations. Lethal effects of the test compound were determined by adding an aqueous solution of Rose Bengal (Fluka, Buchs) (0.1 mg/ml) to an aliquot (1:2, by volume) of the zoosporangium suspension. Nonviable zoosporangia stained deep purple while dormant (viable) ones remained unstained. The method was described in detail by Virányi and Oros (1991).

Antifungal activity. Typically, the conidium suspension of the test fungus was mixed with solutions of *o*-Cba of appropriate concentration and incubated until the conidia started to germinate in the untreated control suspensions. The concentration limits where

no germination at all (MIC) and no effect on the germ tube formation occurred (MTC) were determined by microscopic observation. To asses the antimildew activity on tobacco plants, the leaf sheets were infiltrated with 2.0 mM solution of *o*-Cba 24 h prior to infection with conidia of *E. cichoracearum*. The disease severity (sporulation, size and number of mildew colonies) was compared 8 days after treatment to that found on control plants treated with distilled water.

Antibacterial activity. The appropriate amount of the compound was mixed with the agarized medium of proper composition before pouring into Petri dishes (10 ml medium into a 90 mm diameter dish). Then the agar plates were inoculated with bacterial suspensions using a multipoint inoculator. The intensity of colony growth was evaluated after 24 h incubation at 20–22°C by the following four-grade scale: 0 = no growth, 1 = growth on the limit of visual apperception, 2 = apparent but retarded growth as related to the untreated control, 3 = the colony is not visually distinguishable from the untreated control.

Effect of amino acid supplements on the antimicrobial effect of o-*Cba*

Mineral Agar (MA) medium containing agar-agar (11.0) glucose (5.0), glycerol (2.0), Na-β-glycerophosphate (SIGMA, St. Louis) (0.5), KH_2PO_4 (0.5), $Na_2HPO_4 \cdot 7H_2O$ (0.5), KCl (0.25), $MgSO_4$ sicc. (0.15), $CaCl_2$ sicc. (0.15), $FeSO_4 \cdot 5H_2O$ (0.025), $CuSO_4 \cdot 5H_2O$ (0.005), $MnSO_4 \cdot H_2O$ (0.005), $Ni(NO_3)_2 \cdot 6H_2O$ (0.0001), $CoCl_2 \cdot 6H_2O$ (0.0001) and NH_4NO_3 (1.0) (numbers in brackets refer to concentrations in g/l) was used for testing the effect of organic azote (Bacto Peptone (Oxoid L37), Yeast Extract (Oxoid L21), 5.0 and 3.0 g/l, respectively). The appropriate amino acid (20 mg unless otherwise noted) and a suitable amount of *o*-Cba were mixed with MA medium (10 ml) before pouring into the Petri dishes (90 mm). Inoculation, incubation and assessment were made as described above.

Data analysis. All tests were carried out at least in triplicates. Differences in biological responses were evaluated by the F-probe according to Sváb (1981) using the statistical functions of Excel 5.0 (Microsoft, Redmond, USA).

Results and discussion

The toxicological properties of carborane-containing compounds against various organisms have only been sparsely examined and restricted to medical aspects. For rat liver microsomal enzymes, the unsubstituted *o*-carborane effectively inhibited ethylmorphine-N-demethylase and aniline-hydroxylase and was found to be capable of binding to cytochrome P-450, modifying the spectral properties of this enzyme (Valerino et al., 1974). However, *in vivo* experiments showed that aniline-hydroxylase activity increased slightly, whereas the EM-demethylase and P-450 activities were not altered. In general, the acute toxicity of unsubstituted carboranes and their simple derivatives are in the range of 1,000 to 3,000 mg/kg for rodents (Denisenko et al., 1978; Kahl and Radel, 1994; Wyzlic et al., 1996). It is postulated that due to its extremely high lipophilicity with a logP of +0.99 (Fauchére et al., 1980), *o*-Cba interacts with hydrophobic recognition sites of various receptors or binding sites. In subsequent agriculturally related research, the hydrophobic character of the carboranyl moiety was utilized in the development of potent amphiphilic analogues of the pyrokinin/PBAN insect neuropeptide family capable of penetrating the cuticular surface of a species of moth. The analogues, which featured a replacement of the N-terminal Phe residue of the active core with a 3-carboranylpropionyl cap, elicited efficacious and unnatu-

rally prolonged production of pheromone when applied to the abdominal cuticular surface of female *Heliothis virescens*, the tobacco budworm moth. The potent and long-lasting *in vivo* activity was attributed to stability to endopeptidases and strong receptor binding characteristics (Nachman et al., 1996).

o-Cba treatments at the maximum 2 mM dose (>500 mg/l; Methods A and B) did not cause any visible phytotoxicity symptoms on the tobacco and sunflower plants. *o*-Cba did not exhibit any curative effect on sunflower downy mildew and tobacco powdery mildew nor did it influence the symptoms of mildew diseases.

The mycelial growth of *Pythium* and *Phytophthora* was not influenced by 4 mM of *o*-Cba in the medium (data are not shown). In comparative tests the commercial fungicide metalaxyl inhibited these species by 100 percent at 0.04 mM. The antiperonospora activity of this unnatural amino acid to *P. halstedii* was clearly developmental stage specific (Table 1). The biotrophic stage did not show any sensitivity when the germlings, infested previously with downy mildew fungus, were treated with 0.1 mM (26 mg/l) of *o*-Cba. In comparative treatments, metalaxyl inhibited the disease by 70 ± 14 percent at this dose. All asexual spore forms of *Plasmopara* exhibited remarkable sensitivity to *o*-Cba, with the germinating cystospores being extremely sensitive. This lipophilic compound is able to penetrate into the zoosporangia and inhibits zoospore differentiation. Zoospore motion is more sensitive than plasmalemma semipermeability. The dose-dependent inhibitory effect on motion manifests itself rapidly (5–20 seconds after contact), while an influence on the water permeability of the plasmalemma of zoospores requires more time (15 – >300 seconds after contact depending on the concentration). The

Table 1. Toxicity of *o*-carboranylalanine to various developmental forms of *Plasmopara halstedii*

Developmental forms	*o*-Carboranylalanine				Tridemorph	
	alone		+HIS (1:1 mol/mol)			
	MIC	MTC	MIC	MTC	MIC	MTC
Zoosporangia						
Survival	0.32	0.16	0.67	0.31	3,400	420
Germination	0.078	0.041	0.078	0.041	840	13
Zoospores						
Motion	0.00015	0.000075	0.037	0.000075	6.6	1.6
Plasmalemma	0.019	0.00075	0.078	0.019	26	3.3
Cystospores						
Germination	0.019	0.000075	0.16	0.000075	6.6	0.81

Concentration values are given in μM. *MIC* (minimum inhibitory concentration) is the lowest concentration of the active ingredient (*o*-Cba or tridemorph) in the medium resulting in total (100%) inhibition; *MTC* (maximum tolerated concentration) is the highest dose of the active ingredient not exhibiting observable effects.

sporocid effect of *o*-Cba is comparable to that of tridemorph, a selective zoospore inhibitor fungicide (Oros et al., 1988).

o-Cba exhibited low toxicity to spores of filamentous fungi (Table 2). Differences in the sensitivity of various species were not related to their taxonomic relationships. The actively growing hyphal tips are about five to ten times more sensitive than the initiation of germination of conidia. Curiously, the benomyl resistant *B. cinerea* strain (R) tolerated *o*-Cba at a higher degree than the benomyl sensitive one (W). From a practical point of view the antifungal activity of *o*-Cba is negligible since most current fungicides demonstrate a highly selective lethal effect at <1 mM in this assay system. For example, the broad spectrum activity commercial fungicide prochloraz was lethal to conidia of the benomyl sensitive and tolerant strains of *B. cinerea* at 0.25 and 0.016 mM, and to those of the *M. racemosus* at 0.064 mM. The development of tobacco powdery mildew disease was not affected by *o*-Cba at 2.0 mM (prochloraz was protective at 0.1 mM).

The composition of the medium significantly influenced the toxicity of *o*-Cba to bacteria (Table 3). The antibacterial activity measured on mineral agar free of amino acids was on the order of streptomycin, a widely used agricultural antibiotic. Although there were great differences in the sensitivity of species within each genus indicating that the *o*-Cba sensitivity is not closely

Table 2. Toxicity of carboranylalanine to filamentous fungi

Species		*o*-Carboranylalanine	
Name	Source	MIC	MTC
Zygomycota			
M. racemosus	air	>2.0	>2.0
R. nigricans	sunflower	2.0	0.13
Ascomycota			
A. mali	apple	>2.0	>2.0
A. niger	onion	2.0	0.5
B. allii	onion	2.0	0.13
B. cinerea W	grape	0.5	0.016
B. cinerea R	grape	2.0	0.13
C. cucumerinum	cucumber	2.0	0.13
E. cichoracearum	tobacco	0.5	0.05
F. lycopersici	tomato	>2.0	1.0
G. cingulata	banana	1.0	0.13
M. tulasnei	air	>2.0	0.13
M. roridum	*Primula*	>2.0	0.05
P. oxalicum	cucumber	>2.0	0.05
T. basicola	tobacco	>2.0	0.13
T. harzianum	*Pleurotus*	>2.0	1.0
T. roseum	apple	1.0	0.13

Concentration values are given in mM. *MIC* Minimum inhibitory concentrations of the initiation of the germination of conidia, *MTC* Maximum tolerated concentration of the germ tube development.

Table 3. Comparative toxicity of *o*-carboranylalanine and streptomycin to bacteria

Species		*o*-Carboranylalanine				Streptomycin	
		NA		MA		NA	
Name	Source	MIC	MTC	MIC	MTC	MIC	MTC
Gram negatives							
A. radiobacter	soil	2,500	625	78	2.4	1,000	62
A. tumefaciens	grape	2,500	625	3.7	1.2	125	16
A. tumefaciens	cherry	1,250	625	160	2.4	1,000	62
R. trifolii	clever	1,250	300	19	2.4	1,000	16
X. alfalfae	alfalfa	1,250	300	1,250	2.4	7.5	2.1
X. phaseoli	bean	2,500	300	1,250	2.4	62	4.1
X. malvacearum	cotton	2,500	300	1,250	2.4	250	4.1
X. stewartii	maize	1,250	300	160	2.4	32	2.1
E. atroseptica	potato	625	19	3.7	1.2	250	16
E. herbicola	nut tree	2,500	625	1,250	2.4	32	2.1
E. uredovora	bean rust	2,500	300	1,250	2.4	16	0.69
P. fluorescens	soil	625	160	625	20	16	0.69
P. phaseolicola	lima bean	10,000	300	5,000	625	1,000	32
P. syringae	bean	5,000	160	2,500	2.4	62	4.1
E. coli	human	2,500	300	1,250	2.4	32	2.1
Gram positives							
C. michiganense	tomato	1,250	160	78	1.2	16	2.1
C. flaccumfaciens	sugar beet	1,250	160	625	2.4	62	7.5
C. flaccumfaciens	pelargonium	1,250	160	160	2.4	7.5	0.69
C. flaccumfaciens	tulip	1,250	300	625	1.2	32	4.1
R. fascians	bean	1,250	160	625	2.4	62	0.69
M. luteus	air	625	19	78	1.2	7.5	0.69
S. aureus	human	2,500	300	1,250	2.4	32	2.1
B. subtilis	soil	625	19	3.7	1.2	32	4.1
B. thuringiensis	Thuricide	625	160	3.7	1.2	32	2.1

Concentration values are given in μM. MIC (minimum inhibitory concentration) is the lowest concentration of the active ingredient (*o*-Cba or streptomycin) in the medium resulting in total growth inhibition; MTC (maximum tolerated concentration) is the highest dose of the active ingredient not exhibiting observable effects on the colony formation. *NA* Nutrient Agar, *MA* Mineral Agar (for the composition of culture media see Materials and methods section).

related to their taxonomic relationships, Gram positive species were in general more sensitive than Gram negative ones. The absence of amino acids dramatically increased both the activity of *o*-Cba and the divergence of bacterial response to this unnatural amino acid.

The effect of the peptone, yeast extract and 23 single amino acid nutrients on the *o*-Cba tolerance of bacteria was also investigated. When completing MA with organic azote, bacterial sensitivity to the carborane derivative decreased, and the effect of peptone was more pronounced than that of yeast extract. The high sensitivity of *E. atroseptica*, an important potato pathogen, is particularly interesting because in this case the enrichment of the medium with amino acids did not result in a large decrease of sensitivity. Supplement-

Table 4. Effect of the composition of test media on the inhibition the bacterial growth by *o*-carboranylalanine

Species	Media containing 100 mg/l of *o*-carboranylalanine							
					Single amino acids			
	NA	PA	YA	MA	Asn	Ala	His	Phe
Gram negatives								
A. radiobacter K-84	3	3	2	1	0	0	2	0
A. tumefaciens O	3	3	2	1	0	0	2	0
A. tumefaciens C-58	3	3	2	1	0	0	2	0
R. trifolii	3	3	2	1	0	0	1	0
X. alfalfae	3	2	1	0	0	1	0	0
X. phaseoli	3	3	2	1	0	1	2	0
X. malvacearum	3	3	2	1	0	1	2	0
X. stewartii	3	3	1	0	0	1	1	0
E. atroseptica	3	3	2	1	0	0	1	0
E. herbicola	3	3	1	0	0	0	2	0
E. uredovora	3	3	1	1	1	1	1	0
P. fluorescens	3	3	0	0	0	0	3	0
P. phaseolicola L5	3	3	1	1	0	2	3	0
P. syringae 14	3	3	2	2	0	0	2	0
E. coli	3	2	1	0	0	1	1	0
Gram positives								
C. michiganense	3	2	1	1	0	0	1	0
C. flaccumfaciens 6	3	3	2	1	1	1	2	0
C. flaccumfaciens 8	3	2	0	0	0	0	1	0
C. flaccumfaciens 11	3	2	1	0	0	1	1	0
R. fascians	3	3	2	1	0	1	2	0
S. aureus	3	2	1	0	0	0	0	0
M. luteus	3	3	1	0	0	0	0	0
B. subtilis P-15	3	3	1	0	0	0	1	0
B. thuringiensis	3	2	2	0	0	1	1	0

NA Nutrient Agar, *PA* Peptone Agar, *YA* Yeast Extract Agar, *MA* Mineral Agar. Single amino acids were added to MA at 2 g/l. For the composition of culture media see Materials and methods section.
Antibacterial effect: *0* no growth, *1* growth on the limit of visual apperception, *2* apparent but retarded growth as related to the untreated control, *3* the colony is not distinguishable visually from the untreated control. $LSD_{5\%}$ for medium 0.5 ($F = 399.9 > F_{5\%} = 2.01$) and for bacteria 0.8 ($F = 7.0 < F_{5\%} = 1.57$).

ing MA with single amino acids, only three of them increased the tolerance of bacteria to *o*-Cba. (Table 4) in the following order Asn < Ala < His. However, addition of other single amino acids (Asp, Arg, Cys, Glu, Gln, Gly, His, Ile, Leu, Met, Orn, Phe, Ser, Thr, Tyr, Try, Val, β-Ala, DOPA, GABA) did not protect the bacteria from the toxic effect of *o*-Cba. Similar but developmental stage dependent protective effects of histidine were noticed for *P. halstedii* spores as well. As much as one molar equivalent of L-His in the medium was sufficient enough to markedly decrease the sensitivity of the microorganism tested. Apparently, the unnatural amino acid interferes with

L-His metabolism acting as a classical antimetabolite by a yet unknown mechanism.

Although the observed biological activity of *o*-Cba may be related to the strong lipophilic character of the carboranyl moiety, which might be expected to enhance the penetration into the cell, the antimicrobial mode of action of *o*-Cba requires further studies. The results with histidine are especially interesting, because the metabolism of this amino acid has been suggested as a possible target for pesticide development (Pillimor, 1989). The effect of *o*-Cba in this respect was highly selective indicating possible differences in the organization of the histidine-related metabolism in various organisms, which could serve as a basis for the development of new selective crop protection agents. The large differences in the sensitivity of various taxa of Eu- and Prokaryotes, especially with regard to the highly selective antioomycete activity against *P. halstedii* asexual spores, are also noteworthy. Nevertheless, further studies are warranted in this fied because the metabolism of histidine is less understood than that of the other amino acids (Wallsgrove, 1989). The relatively selective bactericidal effects of this unnatural amino acid suggests that research on this and related carboranyl-based amino acid analogues may provide interesting leads in the search for clinical antibacterial agents and/or in the development of new strategies to ensure the delivery of safe food products to the consumer.

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