Effect of Chloroacetyl Derivatives of *para*-Substituted Phenylalanines on Microbial Antitumor Prescreens

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Abstract □ The chloroacetyl derivatives of four representative *para*-substituted phenylalanines, *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, *N*-chloroacetyl-*p*-nitro-DL-phenylalanine, N^a,N^p-di(chloroacetyl)-*p*-amino-DL-phenylalanine, and *N*-chloroacetyl-*O*-methyl-L-tyrosine, were prepared and tested for growth inhibitory activity in *Lactobacillus casei*. The inhibition with these compounds approximated that of most other inhibitory chloroacetyl amino acids reported previously. However, *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, the most active of these compounds, exhibited an activity approximately that of *N*-chloroacetyl-β-hydroxy-D-norleucine B, the most active *N*-chloroacetyl derivative studied thus far. In view of this finding, the *N*-chloroacetyl derivatives of other *para*-halogenated phenylalanines were prepared and tested. The inhibitory capacity of the *N*-chloroacetyl derivatives of *p*-bromo- and *p*-iodophenylalanine was even greater than that of *p*-chlorophenylalanine, and the order of activity of these compounds increased from the chloroacetyl derivative of *p*-chloro- to that of *p*-bromo- to that of *p*-iodo-DL-phenylalanine. The activity of *N*-chloroacetyl-*p*-bromo- and *N*-chloroacetyl-*p*-iodo-DL-phenylalanine was as great as that of the ring-substituted *N*-benzoylphenylalanines, the most potent *N*-acyl derivatives observed.

Keyphrases □ *N*-Chloroacetyl derivatives of *para*-substituted phenylalanines—tested for growth inhibition in microbial antitumor prescreen, structure–activity relationships □ Amino acid derivatives—*N*-chloroacetyl derivatives of *para*-substituted phenylalanine, structure–activity relationships, growth inhibition in microbial antitumor prescreen □ Structure–activity relationships—*N*-chloroacetyl derivatives of *para*-substituted phenylalanines tested for growth inhibition in microbial antitumor prescreen □ Antineoplastic activity, potential—*N*-chloroacetyl derivatives of *para*-substituted phenylalanines, tested for growth inhibition in microbial antitumor prescreen, structure–activity relationships

Previous studies (1, 2) demonstrated that *N*-chloroacetyl derivatives of certain amino acids and amino acid analogs inhibited growth in a microbial antitumor prescreen where no activity was noted with the parent amino acid. In these studies, a commercially obtained *N*-chloroacetyl-L-tyrosine, even after recrystallization, showed marked inhibitory activity[1]. Whereas most of the other "active" *N*-chloroacetyl amino acids inhibited growth by 20–30% at 4.47 m*M* (2), this preparation of *N*-chloroacetyl-L-tyrosine showed essentially complete inhibition at 0.2 m*M*. Since chloroacetyl-L-phenylalanine exhibited no remarkable activity over that observed for the other inhibitory chloroacetyl amino acids, it was of interest to determine whether the *N*-chloroacetyl derivatives of other *para*-substituted phenylalanines would show the level of activity noted with *N*-chloroacetyl-L-tyrosine.

The *N*-chloroacetyl derivatives of some representative phenylalanines containing *para*-substituted electrophilic or electrophobic groups were prepared and tested for growth inhibition in a microbial antitumor prescreen. This article reports the results of that study.

EXPERIMENTAL

Materials—The free amino acids were obtained from commercial sources and were recrystallized from water–ethanol before use. Purity was ascertained by elemental analysis and by paper chromatography in at least four different solvent systems (2).

Methods—The amino acids were chloroacetylated by the conventional Schotten–Baumann procedure (3). The products were recrystallized from ethyl acetate, and their purity was ascertained by elemental analysis, Van Slyke nitrous acid determination of primary amino nitrogen (4), melting-point determination, and, where applicable, optical rotation measurements (Table I).

Van Slyke nitrous acid determination of primary amino nitrogen (4) was made on 1-ml samples containing an equivalent of 0.250–0.300 mg of the amino nitrogen (when hydrolyzed). Melting points were determined on a melting-point block[2] and are uncorrected. Optical rotation measurements were made on a polarimeter[3] equipped with a sodium lamp, using 100-mm tubes having a 3-mm bore size and a 0.7-ml sample capacity.

Microbial Antitumor Prescreen—The compounds were tested for antitumor activity using a microbial antitumor prescreen described by Foley *et al.* (5). The microbial system used was *Lactobacillus casei* 7469[4] in a riboflavin-supplemented riboflavin assay system. The test compounds were dissolved in water, the solution pH was adjusted to 6 by the addition of 0.1 *N* NaOH, and then the solution was sterilized by passage through an all-glass bacterial filter.

The extent of growth was determined by turbidity measurements in a Klett–Summerson photoelectric colorimeter equipped with a red filter (660 nm) after 19 hr of growth at 37° (1). At least three duplicate determinations were made for each compound. The duplicate values in each determination agreed within ±5 Klett units. The details of the assay were described previously (1).

RESULTS AND DISCUSSION

Table II shows the extent of inhibition caused by the *N*-chloroacetyl derivatives of *para*-substituted phenylalanines assayed in accordance with the microbial antitumor prescreening protocol (5). Initially N^a,N^p-di(chloroacetyl)-*p*-amino-DL-phenylalanine, *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, *N*-chloroacetyl-*O*-methyl-L-tyrosine, and *N*-chloroacetyl-*p*-nitro-DL-phenylalanine were tested. Except for *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, the compounds showed modest inhibition, which was similar to that of most of the other inhibiting chloroacetyl derivatives reported earlier (2), *i.e.*, ~20% at 4.47 m*M*. *N*-Chloroacetyl-*p*-chloro-DL-phenylalanine showed considerably more activity (42% inhibition), approaching a positive test according to the screening protocol, *i.e.*, 50% inhibition at 1 mg/ml (5).

When the inhibitory activities of these compounds were compared on an equimolar basis among themselves (Table III) and with other chloroacetyl derivatives (2), it was found that, with the exception of *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, they were approximately the same as those of the other inhibitory chloroacetyl compounds (2). The activity of *N*-chloroacetyl-*p*-chloro-DL-phenylalanine was approximately that of *N*-chloroacetyl-β-hydroxy-D-norleucine B, the most potent chloroacetyl derivative studied thus far (2, 6).

Because of the striking inhibition exhibited by *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, it was of interest to test the inhibitory activity of the *N*-chloroacetyl derivatives of other *para*-halogenated phenylalanines. Therefore, the *N*-chloroacetyl derivatives of *p*-bromo- and *p*-iodo-DL-phenylalanine were prepared (Table I) and tested for growth

[1] Although subsequent preliminary studies indicated that the inhibitory factor was not *N*-chloroacetyl-L-tyrosine but rather a contaminant present in the commercial product, the results obtained supplement earlier studies on chloroacetylated amino acids (2), especially in view of the relatively potent inhibitions noted with some compounds.

[2] Fisher–Johns.

[3] Rudolph model 80.

[4] American Type Culture Collection, Rockville, Md.

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Table I—Purity Data of *N*-Chloroacetyl Derivatives of *para*-Substituted Phenylalanines

Compound	Melting Point	Optical Rotation[a] $[\alpha]_D^{22-24°}$	Primary Amino Nitrogen	Empirical Formula	Analysis[b], %	Calc.	Obs.
N^a,N^p-Di(chloroacetyl)-*p*-amino-DL-phenylalanine, monohydrate	183–185°	0° (W)	0.7	$C_{13}H_{14}Cl_2N_2O_4$	C	45.63	45.62
					H	4.01	4.35
					Cl	20.19	20.48
					N	8.18	8.22
N-Chloroacetyl-*p*-chloro-DL-phenylalanine	153–155°	0° (A)	1.8	$C_{11}H_{11}Cl_2NO_3$	C	47.85	47.58
					H	4.02	4.35
					Cl	25.68	25.26
					N	5.07	5.33
N-Chloroacetyl-*O*-methyl-L-tyrosine	112–113°	+59.1° (A)	2.3	$C_{12}H_{14}ClNO_4$	C	53.05	53.13
					H	5.19	5.39
					Cl	13.05	12.95
					N	5.16	5.16
N-Chloroacetyl-*p*-nitro-DL-phenylalanine	131°	0° (A)	1.8	$C_{11}H_{11}ClN_2O_5$	C	46.09	45.99
					H	3.87	4.05
					Cl	12.37	12.31
					N	9.77	9.88
N-Chloroacetyl-*p*-bromo-DL-phenylalanine	156–157°	0° (A)	0	$C_{11}H_{11}BrClNO_3$	C	41.21	41.52
					H	3.46	3.44
					N	4.37	4.37
					X[c]	22.12	21.90
N-Chloroacetyl-*p*-iodo-DL-phenylalanine	162–166°	0° (A)	0.8	$C_{11}H_{11}ClINO_3$	C	35.94	36.31
					H	3.02	3.05
					N	3.81	3.65
					X	19.29	19.07

[a] Optical rotation measurements: W = in water plus 1 equivalent of sodium hydroxide, *c*, 2. A = in absolute ethanol, *c*, 2. [b] Elemental analyses were performed by the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. [c] X = percent of total halide as chloride.

inhibitory activity. These compounds also showed considerable activity, some 55% at 1 mg/ml (Table II).

A comparison of activity at equimolar levels showed that both *N*-chloroacetyl-*p*-bromo-DL-phenylalanine and *N*-chloroacetyl-*p*-iodo-DL-phenylalanine inhibited the growth of the test organism to a greater degree than did *N*-chloroacetyl-*p*-chloro-DL-phenylalanine (Table III). At 4.47 m*M*, the concentration at which the activities of the various acyl derivatives were compared, these two acyl derivatives showed almost complete inhibition.

A more detailed study on the relative activities of these *p*-halogenated phenylalanines was made (Fig. 1). The activity increased in order from *N*-chloroacetyl-*p*-chloro-DL-phenylalanine, to *N*-chloroacetyl-*p*-bromo-DL-phenylalanine, to *N*-chloroacetyl-*p*-iodo-DL-phenylalanine. The ID_{50} values of these compounds were 4.3, 2.5, and 1.8 μmoles/ml, respectively. The ID_{50} value of *N*-chloroacetyl-*p*-fluoro-DL-phenylalanine, extrapolated from its inhibition at 4.47 μmoles/ml reported previously (2), was 14.1 μmoles/ml. Thus, the activity of the *N*-chloroacetyl-*p*-halogenated phenylalanine increases with increasing atomic size of the *para*-substituted halogen atom.

In view of the observation that *N*-benzoyl-*p*-chloro-DL-phenylalanine also showed marked inhibition (7, 8), it appears that the *p*-halogenated phenylalanine moiety of the acylated compound exerts an important effect on the final activity of the acylated amino acid. However, the nature of the acyl moiety is also likely to be important since the *N*-benzoyl derivative of *p*-chlorophenylalanine was markedly more active than the corresponding chloroacetyl derivative, the former showing complete inhibition at 4.47 μmoles/ml and the latter showing 48% inhibition at that concentration.

It is unlikely that the activity is due to the hydrolytic release of the parent amino acid, *i.e.*, *p*-chloro-DL-phenylalanine, *p*-bromo-DL-phenylalanine, or *p*-iodo-DL-phenylalanine, since these amino acids showed no appreciable activity when tested in equimolar concentration (Table III). Previous studies (2) also showed that there was no significant hydrolysis of the acylated amino acid during incubation.

The other *para*-substituted phenylalanines showed activity comparable to the active *N*-acylated amino acid analogs (2) and in no way were as inhibitory as the *para*-halogenated derivatives (Tables II and III). Hence, it is unlikely that *para*-substitution *per se* increased activity. Also, since the *p*-nitrophenylalanine derivatives did not show the same degree of activity as the *para*-halogenated phenylalanine derivatives, it is unlikely that the electronegativity of the *para*-substituted species plays a role in conferring the activity.

While the possibility that the observed activity is caused by minute quantities of highly active contaminants is not excluded, it is unlikely since all of the compounds were prepared in these laboratories using identical procedures and were purified using identical reagents for re-

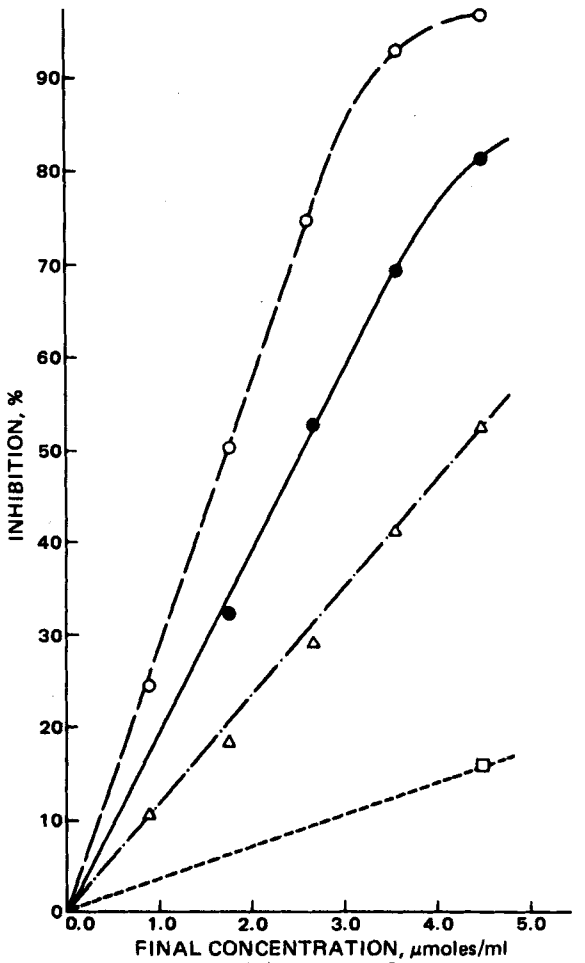

Figure 1—*Comparison of inhibition by* N-*chloroacetyl*-p-*halogenated phenylalanines. Key:* △, N-*chloroacetyl*-p-*chloro*-DL-*phenylalanine;* ●, N-*chloroacetyl*-p-*bromo*-DL-*phenylalanine;* ○, N-*chloroacetyl*-p-*iodo*-DL-*phenylalanine; and* □, N-*chloroacetyl*-p-*fluoro*-DL-*phenylalanine (2).*

Table II—Effect of *N*-Chloroacetyl Derivatives of *para*-Substituted Phenylalanines on the Growth of *L. casei*[a]

Compound	Concentration Equivalent at 1 mg/ml, m*M*	Inhibition[b], % 0.1 mg/ml[c]	0.5 mg/ml[c]	1.0 mg/ml[c]
N-Chloroacetyl-*p*-chloro-DL-phenylalanine	3.62	5	19	42
N-Chloroacetyl-*p*-nitro-DL-phenylalanine	3.49	3	10	18
N^{a},N^{p}-Di(chloroacetyl)-*p*-amino-DL-phenylalanine	3.00	6	13	21
N-Chloroacetyl-*O*-methyl-L-tyrosine	3.68	4	11	20
N-Chloroacetyl-*p*-bromo-DL-phenylalanine	3.12	2	25	56
N-Chloroacetyl-*p*-iodo-DL-phenylalanine	2.72	+3	35	54

[a] For details of assay procedure, see Refs. 1 and 2. [b] Turbidity readings of the inoculated control tubes (containing no test compounds) were 188–196. [c] Final concentration in the assay system.

crystallization. Since widely different inhibitory activities were observed, contaminants producing inhibition would have had to be present in the original compounds and retained during the preparative and purification procedures. This is clearly not the case since compounds that were not chloroacetylated did not inhibit. Therefore, it seems reasonable that the inhibitory activities are intrinsic properties of the newly synthesized compounds and are related principally to the chloroacetylation of the amino group and, secondarily, to the introduction of substituent groups into the aromatic ring of the amino acid.

Table III—Comparison of the Effect of Equimolar Concentrations of *N*-Chloroacetyl Derivatives of *para*-Substituted Phenylalanines on the Growth of *L. casei*[a]

Compound	Inhibition[b], %
N-Chloroacetyl-*p*-chloro-DL-phenylalanine	48
N-Chloroacetyl-*p*-nitro-DL-phenylalanine	19
N^{a},N^{p}-Di(chloroacetyl)-*p*-amino-DL-phenylalanine	22
N-Chloroacetyl-*O*-methyl-L-tyrosine	20
N-Chloroacetyl-DL-phenylalanine	20
N-Chloroacetyl-*p*-bromo-DL-phenylalanine	86
N-Chloroacetyl-*p*-iodo-DL-phenylalanine	93
p-Bromo-DL-phenylalanine[c]	+3
p-Chloro-DL-phenylalanine[c]	0
p-Iodo-DL-phenylalanine[c]	0

[a] Maximum growth in inoculated control tubes (containing no test compound) was 186–196 Klett units. [b] Concentration was 4.47 μmoles/ml and was the final concentration of the assay system. [c] Free amino acid.

In studies of this nature, where empirical relationships of inhibitory capabilities are sought, there is little information regarding the mechanism of action. To study the mechanism of action, more sophisticated experiments are required.

The activity of the *N*-benzoyl-*p*-bromo and *N*-benzoyl-*p*-iodophenylalanine is being studied and compared with the corresponding *N*-chloroacetyl compounds.

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COMMUNICATIONS

Nasal Absorption of Natural Contraceptive Steroids in Rats—Progesterone Absorption

Keyphrases □ Progesterone—nasal absorption compared with that following intravenous and intraduodenal administration, rats □ Absorption, nasal—progesterone, compared with absorption following intravenous and intraduodenal administration, rats □ Contraceptives, natural—progesterone, nasal absorption compared with that following intravenous and intraduodenal administration, rats □ TLC—identification of unchanged progesterone following nasal administration to rats, compared with intravenous and intraduodenal administration

To the Editor:

Contraceptive natural steroids such as progesterone and estradiol are ineffective when given orally due to extensive metabolism in the GI tract during absorption and first-pass metabolism (1, 2). Thus, highly potent and potentially harmful synthetic steroids are currently being used in oral dosage forms.

To enhance progesterone bioavailability from nonparenteral routes, the nasal route was examined. Previous studies showed that propranolol is absorbed efficiently from the nasal mucosa of rats and dogs (3, 4). Sprague–Dawley male rats, 300 g, were anesthetized with pentobarbital sodium (50 mg/kg). For each dose and for each administration route, four to six rats were used.

For nasal administration, three doses of 50, 100, and 150 μg of [4-^{14}C]progesterone (8 μCi) in 0.1 ml of 1% polysorbate 80–saline solution were administered to the nasal cavity of each rat by a micropipet according to the procedure described previously (3). For intravenous administration, the same doses were injected through the femoral vein. For intraduodenal administration, the abdomen was opened by a midline incision, and the 50-μg dose in 0.1 ml of 1% polysorbate 80–saline solution was injected directly through the duodenum.

After administration, 0.2 ml of blood was sampled periodically from the femoral aorta. The blood sample was

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