Structure–Activity Relationships Among Substituted *N*-Benzoyl Derivatives of Phenylalanine and Its Analogs in a Microbial Antitumor Prescreen I: Derivatives of *o*-Fluoro-DL-phenylalanine

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Abstract □ Twelve derivatives of *o*-fluoro-DL-phenylalanine containing fluorine, chlorine, methoxy, and nitro radicals in various positions of the aromatic ring of the benzoyl group were prepared and tested in a *Lactobacillus casei* system. It was found that most substitutions in the benzoyl phenyl ring resulted in a compound exhibiting greater growth-inhibiting activity than the nonsubstituted benzoyl-*o*-fluorophenylalanine. The greatest activity was observed in the *ortho*-substituted fluoro compound and the *meta*- and *para*-substituted chloro and nitro compounds. With the methoxy group, the position of substitution appeared unimportant, since all three methoxy isomers exhibited essentially equal inhibition. Nitro substitution in the *ortho* position had a protective effect in that the product was less active than the unsubstituted benzoyl-*o*-fluoro-DL-phenylalanine.

Keyphrases □ Structure–activity relationships—substituted *N*-benzoyl derivatives of phenylalanine, microbial antitumor prescreen □ Phenylalanine—substituted *N*-benzoyl derivatives, microbial antitumor prescreen □ Antitumor agents, potential—microbial antitumor prescreen, substituted *N*-benzoyl phenylalanine derivatives

Previous studies on the effect of *N*-acylated amino acids and amino acid analogs on the growth of *Lactobacillus casei* in an antitumor prescreen indicate that certain acyl groups could activate an otherwise inert amino acid or amino acid analog to inhibit the growth of the organism (1–4). *N*-Chloroacetyl (1–3) and *N*-trifluoroacetyl (4) derivatives of certain amino acids exhibited modest but significant inhibition. However, among the acyl amino acids studied, the *N*-benzoyl derivatives showed the greatest inhibition, especially the benzoyl derivatives of phenylalanine analogs (5, 6). The nature and the position of the substituent in the aromatic ring of phenylalanine are apparently of some importance in the action, since substituents at various positions in the ring resulted in compounds of different activity. Of the seven benzoyl derivatives of ring-substituted phenylalanine and phenylalanine analogs studied earlier, benzoyl-*p*-chloro- and benzoyl-*m*-fluorophenylalanine showed the greatest inhibition (5, 6). Among the *N*-chloroacetyl derivatives of *p*-halophenylalanines the activity decreased in order from the *p*-iodo through the *p*-bromo and the *p*-chloro to the *p*-fluoro derivatives (7).

This study attempted to find out whether substituents in the aromatic ring of the benzoyl moiety would similarly affect the inhibitory activity of these compounds and whether more potent inhibitors could be devised by combination of the appropriate substituted benzoyl and phenylalanine moieties. Thus, a systematic study was undertaken to observe the effect of varying the substituents (a) to the benzoyl moiety while keeping the amino acid portion constant, and (b) to the amino acid while keeping the benzoyl moiety unchanged.

In this study, the amino acid moiety was *o*-fluoro-DL-phenylalanine, and the aroyl part included 12 ring-substituted benzoyl derivatives[1].

EXPERIMENTAL

o-Fluoro-DL-phenylalanine was obtained commercially[2]. Its purity was checked by paper chromatography in four separate solvent systems (*cf.* Ref. 2), elemental analysis, and by nitrous acid gasometric analysis of primary amino nitrogen (8). When necessary it was recrystallized from water–ethanol. The acylating agents were obtained from commercial sources and purified, by distillation or recrystallization when necessary.

The acyl derivatives were prepared by the Schotten–Baumann procedure (*cf.* Ref. 9). The liquid acylating agents were added in 1.2 mole excess directly into the reaction mixture in six equal portions over a 1-hr period. Solids were added as an ethyl acetate solution, also in six equal portions over a 1-hr period. The reaction in most cases was carried out between 0 and 5°. However, when the acylating agent solidified at that temperature, the reaction was carried out at 10°.

The purity of the prepared acyl derivatives was ascertained by elemental analysis, melting point determination, and by nitrous acid gasometric determination of primary amino nitrogen (8) (Table I).

For the microbiological assay, the acyl compounds were dissolved in one equivalent of sodium hydroxide. In some cases, warming was required to dissolve the compound. At this point, the pH of the test solution was ~6.

A modification was made on the sterilization procedure of the test compounds so that a larger number of compounds could be tested each time. The all-glass filtration procedure for sterilization was replaced with one using sterilizing filter units[3] (0.22-μm pores) attached to plastic syringes[4].

The assay medium was a riboflavin-supplemented riboflavin assay medium[5] containing 0.03 μg of riboflavin[6]/ml in the final assay system.

The system was incubated at 37.5° for 19 hr, and then bacteria growth was terminated by immersing the tubes in boiling water for 10 min. After the suspension was cooled in an ice bath for 10 min, the growth was determined turbidimetrically on a photoelectric colorimeter[7] equipped with a red filter (660 nm).

The organism used was *Lactobacillus casei* 7469[8], which was carried on agar[9], subcultured bimonthly, and transferred to a broth[9] for the preparation of the inoculum. The inoculum was a 1:20 diluted suspension of the washed bacteria in 10 ml of normal saline. Details of the assay procedure were described previously (1).

The extent of growth in the inoculated control (containing no test compound), the inhibitor control (containing 6-mercaptopurine[10]), and in some of the compounds previously studied, varied with different lots

[1] This paper is part of a continuing study of the effect of *N*-acylation of amino acids and of amino acid analogs on the growth of microorganisms used as a prescreen for antitumor activity and is the first of a series of papers describing the inhibitory effect of substituted benzoyl derivatives of various phenylalanine analogs.

[2] ICN Pharmaceuticals, Cleveland, OH 44128.

[3] Millex-GS, Millipore Corp., Bedford, MA 01730.

[4] Plastipak disposable syringes, Becton-Dickinson and Co., Rutherford, NJ 07070.

[5] Difco B325, Difco Laboratories, Detroit, Mich.

[6] Calbiochem-Behring Corp., San Diego, CA 92112.

[7] Klett-Summerson photoelectric colorimeter, Arthur H. Thomas Co., Philadelphia, PA 19105.

[8] The American Type Culture Collection, Rockville, MD 20852.

[9] Micro assay culture agar (Difco B319), Micro inoculum broth (Difco B320) Difco Laboratories, Detroit, Mich.

[10] Sigma Chemical Co., St. Louis, MO 63178.

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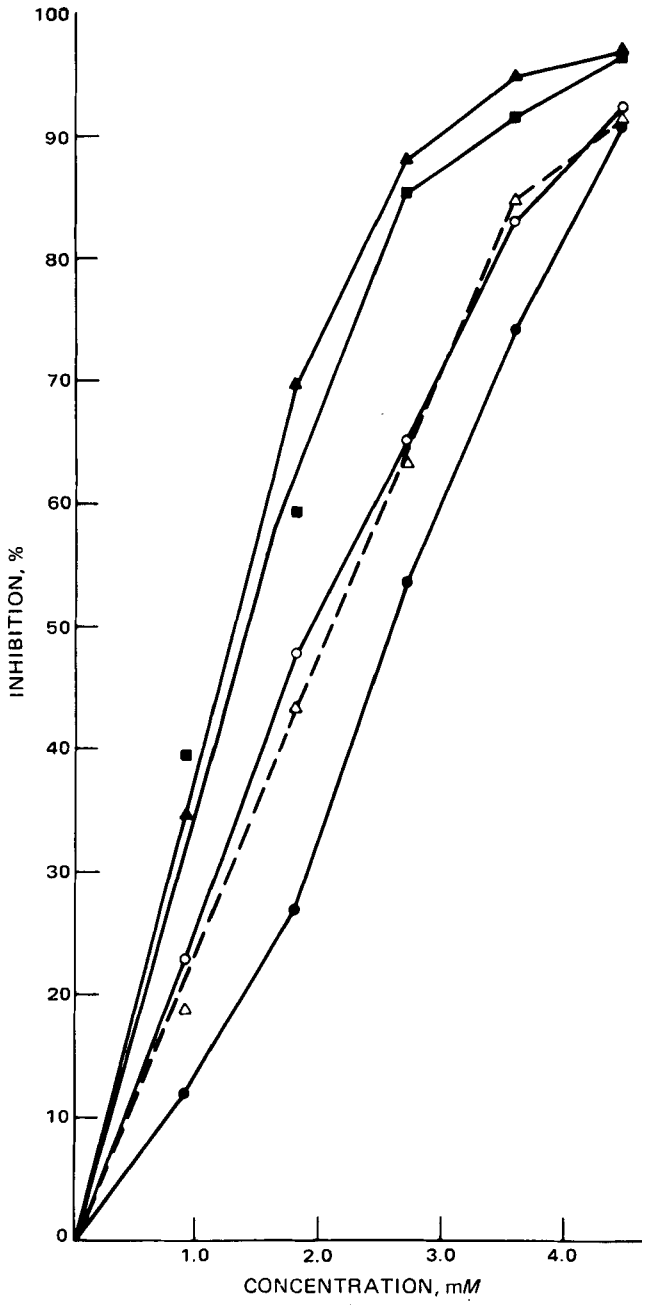

Figure 1—*Inhibition curves of some substituted benzoyl-o-fluoro-DL-phenylalanines: determination of ID_{50}. Key:* ●, *o-fluorobenzoyl-o-fluoro-DL-phenylalanine;* ▲, *m-chlorobenzoyl-o-fluoro-DL-phenylalanine;* ■, *p-chlorobenzoyl-o-fluoro-DL-phenylalanine;* ○, *m-nitrobenzoyl-o-fluoro-DL-phenylalanine; and* △, *p-nitrobenzoyl-o-fluoro-DL-phenylalanine.*

of the riboflavin assay medium used. Therefore, in this study and all subsequent studies of this series, the comparison of the extent of growth was made using medium of the same lot. In addition, the pH of the assay medium was adjusted uniformly to 6.40 by the addition of an appropriate amount of 0.5 *N* HCl before addition of the test compounds. The pH of the final system, determined on an aliquot of the medium containing an equivalent amount of the test compounds as the experimental tubes, was 6.20, and was within 0.1 pH units of the control tubes containing no test compounds.

RESULTS AND DISCUSSION

Most of the substituted *N*-benzoyl derivatives of *o*-fluoro-DL-phenylalanine showed considerable inhibitory activity. Of the 12 compounds tested, nine showed inhibition of ≥50% (Table II). These compounds may be considered to be positive according to the protocol of the microbial antitumor prescreen (10), and will be tested further in mammalian tumor systems.

Of the remaining three compounds, *m*-fluorobenzoyl- and *p*-fluorobenzoyl-*o*-fluoro-DL-phenylalanine, though slightly less active, nevertheless showed almost as much inhibiting activity as the more active chloroacetyl (2, 3) and trifluoroacetyl (4) derivatives studied earlier. The *o*-nitrobenzoyl compound showed about the same activity as most of the active *N*-chloroacetyl amino acids studied previously (3).

Comparison of activity within each group of derivatives (Table II) showed that the nature and the position of the substituents in the benzoyl ring were important to the inhibitory capacity of the compounds. For example, the substitution of a fluorine atom at the *ortho* position in the benzoyl ring of the fluorobenzoyl-*o*-fluoro-phenylalanines resulted in the most active derivative of this series, showing 69% inhibition at 1 mg/ml. Though not as pronounced, fluorine substitution at the *meta* or the *para* position in the benzoyl ring yielded derivatives of appreciable activity (39–47%), the *meta* isomer being slightly more active than the *para* isomer.

With the chlorobenzoyl and nitrobenzoyl derivatives, the *meta* and

Table I—Purity of Benzoyl Ring Substituted Derivatives of *o*-Fluoro-DL-phenylalanine [a]

o-Fluoro-DL-phenylalanine Derivative	Melting Point [b]	Empirical Formula	Analysis [c], %		
				Calc.	Found
o-Fluorobenzoyl	167–168	$C_{16}H_{13}F_2NO_3$	C	62.95	62.72
			H	4.29	4.41
			N	4.59	4.47
			F	12.45	12.86
m-Fluorobenzoyl	168–169	$C_{16}H_{13}F_2NO_3$	C	62.95	62.38
			H	4.29	4.25
			N	4.59	4.35
			F	12.45	12.63
p-Fluorobenzoyl	224	$C_{16}H_{13}F_2NO_3$	C	62.95	62.64
			H	4.29	4.46
			N	4.59	4.61
			F	12.45	12.69
o-Chlorobenzoyl	172–175	$C_{16}H_{13}ClFNO_3$	C	59.73	59.73
			H	4.07	4.11
			N	4.35	4.39
			Cl	11.02	11.13
			F	5.90	5.62
m-Chlorobenzoyl	160–162	$C_{16}H_{13}ClFNO_3$	C	59.73	59.97
			H	4.07	4.16
			N	4.35	4.39
			Cl	11.02	11.17
			F	5.90	6.16
p-Chlorobenzoyl	208–209	$C_{16}H_{13}ClFNO_3$	C	59.73	59.44
			H	4.07	4.07
			N	4.35	4.45
			Cl	11.02	11.02
			F	5.90	5.97
o-Methoxybenzoyl	119–121	$C_{17}H_{16}FNO_4$	C	64.35	64.42
			H	5.08	4.95
			N	4.41	4.42
			F	5.99	5.89
m-Methoxybenzoyl	154–157	$C_{17}H_{16}FNO_4$	C	64.35	63.99
			H	5.08	5.28
			N	4.41	4.53
			F	5.99	6.11
p-Methoxybenzoyl (anisoyl)	168–170	$C_{17}H_{16}FNO_4$	C	64.35	64.85
			H	5.08	5.20
			N	4.41	4.68
			F	5.99	5.63
o-Nitrobenzoyl	177–182	$C_{16}H_{13}FN_2O_5$	C	57.84	57.55
			H	3.94	4.09
			N	8.43	8.30
			F	5.72	5.71
m-Nitrobenzoyl	162–164	$C_{16}H_{13}FN_2O_5$	C	57.84	57.59
			H	3.94	4.11
			N	8.43	8.31
			F	5.72	5.65
p-Nitrobenzoyl	179–182	$C_{16}H_{13}FN_2O_5$	C	57.84	57.58
			H	3.94	3.88
			N	8.43	8.32
			F	5.72	5.83

[a] Van Slyke nitrous acid determination of primary amino nitrogen (8) made on a 1-ml sample containing an equivalent of 0.25–0.30 mg of amino nitrogen (when hydrolyzed) yielded no detectable quantity of nitrogen. [b] Melting points were determined on a Fisher-Johns melting point block and are uncorrected. [c] Elemental analyses were performed by the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institute of Health, Bethesda, Md.

Table II—Effect of Benzoyl Ring Substituted Derivatives of *o*-Fluorobenzoyl-DL-phenylalanine on the Growth of *L. casei* 7469 [a]

o-Fluoro-DL-phenylalanine Derivative	m*M* Equivalent at 1 mg/ml	Inhibition[b], % 0.1 mg/ml[c]	0.5 mg/ml[c]	1.0 mg/ml[c]
o-Fluorobenzoyl	3.28	5	22	69
m-Fluorobenzoyl	3.28	0	18	47
p-Fluorobenzoyl	3.28	0	11	39
o-Chlorobenzoyl	3.11	6	27	51
m-Chlorobenzoyl	3.11	5	55	90
p-Chlorobenzoyl	3.11	9	57	88
o-Methoxybenzoyl	3.15	6	31	53
m-Methoxybenzoyl	3.15	1	21	53
p-Methoxybenzoyl-(anisoyl)	3.15	5	28	54
o-Nitrobenzoyl	3.01	1	9	17
m-Nitrobenzoyl	3.01	0	36	68
p-Nitrobenzoyl	3.01	0	28	70
Benzoyl	3.48	2	12	31

[a] For details of assay, see Ref. 1. [b] Turbidity readings of the inoculated control tubes (containing no test compound) were 182–194 Klett units. At least three duplicate determinations were made for each compound. The duplicate values in each determination agreed within ±5 Klett units; *e.g.*, the standard deviation of a compound showing a mean value of ~20% inhibition was 2.7 and the standard error was 0.95. [c] Final concentration in assay system.

the *para* isomers were the most active of the three isomers. Hence, *m*-chlorobenzoyl- and *p*-chlorobenzoyl-*o*-fluoro-DL-phenylalanine showed nearly complete inhibition at 1 mg/ml, while the corresponding *o*-derivative showed an inhibition of 51%, and *m*-nitrobenzoyl- and *p*-nitrobenzoyl-*o*-fluoro-DL-phenylalanine exhibited activity of ~70%, while the corresponding *ortho* isomer showed only 17% inhibition.

When the substituent was a methoxy group, the position of substitution in the benzoyl ring appeared unimportant since all three isomers of methoxybenzoylphenylalanine exhibited about the same degree of inhibition (54%).

To allow comparison of relative activities, the degree of inhibition of each compound at 4.47 m*M* was determined (Table III). This concentration is equivalent to 1 mg/ml final concentration in the assay system of *N*-chloroacetyl-D-β-hydroxynorleucine B, the most active *N*-acyl alicyclic amino acid studied earlier (2).

The most active benzoyl ring substituted *o*-fluoro-DL-phenylalanines were *o*-fluorobenzoyl, *m*-chlorobenzoyl, *p*-chlorobenzoyl, *m*-nitrobenzoyl, and *p*-nitrobenzoyl derivatives, all of which showed essentially complete inhibition at 4.47 m*M*.

An inhibition curve was constructed for each of these compounds (Fig. 1). This showed that the most active of these compounds were *m*-chlorobenzoyl- and *p*-chlorobenzoyl-*o*-fluoro-DL-phenylalanine, with ID_{50} values of 1.30 and 1.40, respectively. The next active compounds were the *m*-nitrobenzoyl and *p*-nitrobenzoyl derivatives (ID_{50} 1.95 and 2.10, respectively), and the least active was *o*-fluorobenzoyl-*o*-fluoro-DL-phenylalanine (ID_{50} 2.60). Thus, when the amino acid moiety was phenylalanine containing an electronegative group, *i.e.*, fluorine, in the *ortho* position, a large electronegative group such as chlorine or a nitro group in the *meta* position of the benzoyl ring appeared to increase the inhibitory capacity.

Table III—Comparison of the Effect of Equimolar Concentrations of Benzoyl Ring Substituted Derivatives of *o*-Fluoro-DL-phenylalanine on the Growth of *L. casei* 7469 [a]

Derivative of *o*-Fluoro-DL-phenylalanine	Inhibition[b], %
o-Fluorobenzoyl	94
m-Fluorobenzoyl	61
p-Fluorobenzoyl	54
o-Chlorobenzoyl	66
m-Chlorobenzoyl	97
p-Chlorobenzoyl	96
o-Methoxybenzoyl	76
m-Methoxybenzoyl	75
p-Methoxybenzoyl (anisoyl)	74
o-Nitrobenzoyl	28
m-Nitrobenzoyl	92
p-Nitrobenzoyl	92
Benzoyl	44

[a] Maximum growth in inoculated control tube (containing no test compound) was 182–194 Klett units. For explanation of extent of variation of values, see Table II footnotes. [b] Concentration was 4.47 m*M* and was the final concentration in the assay system. For details of assay, see Ref. 1.

Table IV—Effect of Substituted Benzoic Acids on the Growth of *L. casei* 7469 [a]

Substituted Benzoic Acid	Inhibition[b], %
o-Fluorobenzoic Acid	6
m-Fluorobenzoic Acid	10
p-Fluorobenzoic Acid	7
o-Chlorobenzoic Acid	7
m-Chlorobenzoic Acid	32
p-Chlorobenzoic Acid	32
o-Methoxybenzoic Acid	3
m-Methoxybenzoic Acid	10
p-Methoxybenzoic Acid (anisic acid)	6
o-Nitrobenzoic Acid	9
m-Nitrobenzoic Acid	30
p-Nitrobenzoic Acid	34

[a] See Table II footnotes for details of the assay. [b] Concentration was 4.47 m*M*.

Most of the other seven derivatives showed striking activity (54–76% inhibition). This inhibition was considerably greater than that of compounds with no substituent on the benzoyl ring; benzoyl-*o*-fluoro-DL-phenylalanine inhibited 44%.

The activity of *o*-nitrobenzoyl-*o*-fluoro-DL-phenylalanine (28%), the least active of all the derivatives of the series, was less than that of the unsubstituted benzoyl derivative, *N*-benzoyl-*o*-fluoro-DL-phenylalanine. However, its inhibition was as great as most of the inhibiting chloroacetyl derivatives, which inhibited ~15–35% (3).

Thus, except for *o*-nitrobenzoyl-*o*-fluoro-DL-phenylalanine, all substituents in the benzoyl ring have an inhibitory enhancing effect, whatever the electronic nature of the substituent (*i.e.*, electron-withdrawing or electron-donating).

The position of these substituents in the benzoyl ring appeared to be important, especially when the substituent was strongly electronegative, such as in the chlorine, fluorine, and nitro groups. The data are not yet sufficient to draw conclusions about the relationship between the substituent of the benzoyl ring and that of the phenyl ring of phenylalanine. Only a more restrictive observation applying only to the derivatives of *o*-fluoro-DL-phenylalanine could be made. Subsequent studies will give further information on the role of the amino acid moiety in the inhibitory capacity.

The inhibition is not likely due to the release of hydrolytic products of the substituted benzoyl amino acids, *i.e.*, the release of the ring-substituted benzoic acid or the *o*-fluoro-DL-phenylalanine. Except for *m*-chlorobenzoic acid, *p*-chlorobenzoic acid, *m*-nitrobenzoic acid, and *p*-nitrobenzoic acid, all of which showed ~32–34% inhibition, the substituted benzoic acid had essentially no inhibitory activity (Table IV). For those substituted benzoic acids showing some activity, the benzoyl amino acids containing these groups were decidedly more active than the non-substituted benzoic acid. *o*-Fluoro-DL-phenylalanine was shown to be inactive in this assay system (3). Furthermore, it was demonstrated previously (3) that no notable degree of hydrolysis occurred during incubation.

There is insufficient data to propose a possible inhibition mechanism from these experiments. However, based on earlier studies on proteases (11, 12) where aromatic ring-rich dipeptides were shown to be superior substrates of some of these enzymes, and knowing that proteolytic enzymes are commonly produced by microorganisms, it is reasonable to speculate on the involvement of these aroyl phenylalanine analogs in the inhibition of such enzymes.

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