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N-Benzoyl Derivatives of Amino Acids and Amino Acid Analogs as Growth Inhibitors in Microbial Antitumor Screen

THEODORE T. OTANI ˣ and MARY R. BRILEY

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Abstract □ Twenty-seven *N*-benzoyl derivatives of amino acids and amino acid analogs were prepared and tested for growth-inhibitory activity in a microbial antitumor screen. Of these, 19 showed some inhibitory capacity, from a modest 13% to a potent 96% at 1 mg/ml. The activities of the "modest" inhibitors were comparable to those of most inhibitory chloroacetyl and trifluoroacetyl derivatives reported earlier. The intermediate inhibitors were as active as *N*-chloroacetyl-β-hydroxy-D-norleucine isomer B, the most active acyl derivative noted previously. The most active compounds in this study were *N*-benzoyl-*p*-chloro-DL-phenylalanine and *N*-benzoyl-*m*-fluoro-DL-phenylalanine, which inhibited the test organism almost completely under the assay conditions.

Keyphrases □ *N*-Benzoyl amino acid derivatives—antineoplastic activity, potential, inhibition of microbial antitumor screen, structure–activity relationships □ Amino acid derivatives—*N*-benzoyl, potential antineoplastic activity, inhibition of microbial antitumor screen, structure–activity relationships □ Antineoplastic agents, potential—*N*-benzoyl amino acid derivatives, inhibition of microbial antitumor screen, structure–activity relationships □ Structure–activity relationships—*N*-benzoyl amino acid derivatives, inhibition of microbial antitumor screen

Studies of possible antimetabolic effects of novel β-hydroxyamino acids produced evidence that an otherwise inert amino acid could, upon *N*-chloroacetylation, actively inhibit growth of a microbial antitumor screen (1). This study was extended to include the *N*-chloroacetyl (2) as well as the *N*-acetyl (2), *N*-propionyl (2), and *N*-trifluoroacetyl (3) derivatives of other amino acids, both natural and unnatural. Both the amino acid and the *N*-acyl moieties of the acyl amino acid were important in imparting the growth-inhibitory characteristic to the compound, since only certain amino acids and certain acyl groups were capable of doing so (2).

One deterrent to using amino acid analogs as antimetabolites in mammalian systems is their high toxicity (4–7). In view of the finding that there is an alteration in biological properties, especially cytotoxicity, upon *N*-acylation (2), more potent inhibitors might be prepared by attachment of acyl groups other than those studied previously. Since a more lipophilic acyl group might increase the mobility of these compounds in a lipoid milieu such as the cellular membranes in mammalian systems, a benzoyl derivative series was prepared. These compounds were tested for growth inhibition in a microbial system selected specifically as an antitumor screen. Although the ultimate objective of these studies is to find compounds for cancer therapy, the immediate aim has been to improve the growth-inhibitory properties of the compounds in the screen described earlier.

The present paper reports the results of these studies.

EXPERIMENTAL

The free amino acids except the β-hydroxynorleucines, which were prepared in this laboratory (8, 9), were obtained commercially and were recrystallized from water–ethanol before use. Some *N*-benzoyl derivatives were obtained commercially and were recrystallized from ethanol–water before use. Others, especially those of amino acid analogs, were prepared by the Schotten–Baumann procedure (10) and were recrystallized from ethanol or ethanol–water. The sources of the amino acids and of the benzoyl derivatives are shown in Table I.

The purity of the amino acids and of the benzoyl derivatives was ascertained by: (*a*) Van Slyke nitrous acid determination of primary amino nitrogen (11), (*b*) optical rotation measurement, where applicable, and (*c*) elemental analysis. In addition, the purity of the free amino acids was checked by paper chromatography in at least four different solvent systems (8), and that of the benzoyl derivatives was checked by melting-point determination (Table I).

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Table I—Purity of *N*-Benzoyl Derivatives [a]

N-Benzoyl Derivative	Source [b]	Melting Point		Optical Rotation [d] $[\alpha]_D^{21-24}$		Concentration and Solvent	Empirical Formula	Analysis [e], %		
		Reported	Observed [c]	Reported	Observed				Calc.	Obs.
L-Alanine	1	150–151° [f]	150–151°	+37.1° [f]	+36.1°	9.3%, water + 1 eq of potassium hydroxide	$C_{10}H_{11}NO_3$	C	62.17	61.93
								H	5.74	6.03
								N	7.25	7.24
DL-Allylglycine	1	—	112–114°	—	0	2% ethanol	$C_{12}H_{13}NO_3$	C	65.74	65.98
								H	5.98	6.24
								N	6.39	6.44
L-Arginine (α-mono), hemihydrate	2	298° dec. [f]	290°	−8.1° [f]	−8.5°	1.2%, water + 1 eq of hydrochloric acid	$C_{13}H_{19}N_4O_{3\frac{1}{2}}$	C	54.34	54.15
								H	6.67	6.65
								N	19.50	19.48
L-Aspartic acid, monohydrate	3	184–185° [f]	182°	+37.4° [f]	+38.0° [g]	9%, water + 2 eq of potassium hydroxide	$C_{11}H_{13}NO_6$	C	51.76	51.85
								H	5.13	4.81
								N	5.90	5.67
p-Chloro-DL-phenylalanine	1	—	178–180°	—	0	2%, ethanol	$C_{16}H_{14}ClNO_3$	C	63.27	62.98
								H	4.64	4.77
								N	4.61	4.58
o-Fluoro-DL-phenylalanine	1	—	189–190°	—	0	2%, ethanol	$C_{16}H_{14}FNO_3$	C	66.89	67.06
								H	4.91	5.07
								N	4.88	5.14
m-Fluoro-DL-phenylalanine	1	—	188–189°	—	0	2%, water + 1 eq of sodium hydroxide	$C_{16}H_{14}FNO_3$	C	66.89	66.80
								H	4.91	4.91
								N	4.88	4.82
p-Fluoro-DL-phenylalanine	1	—	166–168°	—	0	2%, ethanol	$C_{16}H_{14}FNO_3$	C	66.89	67.21
								H	4.91	4.91
								N	4.88	5.16
L-Glutamic acid, hemihydrate	4	137–139° [f]	137–138°	+17.2° [f]	+16.8° [g]	10%, water + 2 eq of potassium hydroxide	$C_{12}H_{14}NO_{5\frac{1}{2}}$	C	55.38	55.56
								H	5.42	5.24
								N	5.38	5.20
Glycine	4	187.5° [f]	192°	—	—	—	$C_9H_9NO_3$	C	60.33	60.60
								H	5.06	4.93
								N	7.82	7.86
L-Histidine, monohydrate	5	249° [f]	243° dec.	−47.4° [f]	−46.9°	6.8%, 0.5 *M* hydrochloric acid	$C_{13}H_{15}N_3O_4$	C	56.31	56.12
								H	5.45	5.59
								N	15.15	14.85
β-Hydroxy-DL-norleucine A	1	—	153–154°	—	—	—	$C_{13}H_{17}NO_4$	C	62.14	62.09
								H	6.82	6.98
								N	5.58	5.64
β-Hydroxy-DL-norleucine B [h]	1	—	189–190°	—	—	—	$C_{13}H_{17}NO_4$	C	62.14	61.55
								H	6.82	7.02
								N	5.58	6.10
L-Leucine	3	105–107° [f]	105–107°	+6.6° [f]	+6.5°	9.5%, 0.5 *M* potassium hydroxide	$C_{13}H_{17}NO_3$	C	66.36	66.55
								H	7.28	7.39
								N	5.95	6.01
L-Lysine (α-mono)	3	250° dec. [f]	258° dec.	+21.6° [f]	+20.4°	2%, water + 1 eq of sodium hydroxide	$C_{13}H_{18}N_2O_3$	C	62.38	62.21
								H	7.24	7.40
								N	11.19	11.31
DL-Methionine	1	151° [f]	150–151°	—	—	—	$C_{12}H_{16}NO_3S$	C	56.67	56.42
								H	6.34	6.19
								N	5.51	5.51
L-Methionine	1	—	98°	—	−14.6°	2%, ethanol	$C_{12}H_{16}NO_3S$	C	56.67	56.42
								H	6.34	6.18
								N	5.51	5.46
p-Nitro-L-phenylalanine	1	—	205–208°	—	−72.6°	2%, ethanol	$C_{16}H_{14}N_2O_5$	C	61.14	60.50
								H	4.49	4.69
								N	8.91	8.62
DL-Norleucine	1	—	137°	—	0	2%, ethanol	$C_{13}H_{17}NO_3$	C	66.36	66.55
								H	7.28	7.26
								N	5.95	5.85
DL-Norvaline	1	—	152–154°	—	—	—	$C_{12}H_{15}NO_3$	C	65.14	64.98
								H	6.83	6.94
								N	6.33	6.40
L-Phenylalanine, hemihydrate	3	—	141–143°	—	+10.0°	2%, 1 *M* potassium hydroxide	$C_{16}H_{16}NO_{3\frac{1}{2}}$	C	69.05	69.09
								H	5.80	5.82
								N	5.03	4.94
β-2-Thienyl-DL-alanine	1	—	184°	—	0	2%, water	$C_{14}H_{13}NO_3S$	C	61.07	61.12
								H	4.76	4.92
								N	5.09	4.79
β-3-Thienyl-DL-alanine, hemihydrate	1	—	167–168°	—	—	—	$C_{14}H_{14}NO_{3\frac{1}{2}}S$	C	59.14	59.46
								H	4.96	4.68
								N	4.93	5.21
L-Threonine	1	143–144° [f]	141–143°	+27.1°	+26.1°	0.8%, water	$C_{11}H_{13}NO_4$	C	59.19	58.99
								H	5.87	5.86
								N	6.28	6.11
L-Tryptophan, hemihydrate	3	104–105° [f]	105–107°	—	−7.4°	6%, ethanol	$C_{18}H_{17}N_2O_{3\frac{1}{2}}$	C	68.13	68.06
								H	5.40	5.57
								N	8.83	8.87
L-Tyrosine ethyl ester	5	—	207–208°	—	+24.3°	2%, ethanol	$C_{18}H_{19}NO_4$	C	68.99	68.72
								H	6.11	6.11
								N	4.47	4.49
L-Valine	3	131–132° [f]	132–134°	+21.8°	+21.2°	4.9%, ethanol	$C_{12}H_{15}NO_3$	C	65.14	64.91
								H	6.83	6.95
								N	6.33	6.32

[a] Van Slyke nitrous acid determination of primary amino nitrogen (11) made on a 1-ml sample containing an equivalent of 0.3 mg of amino nitrogen (when hydrolyzed) yielded no detectable quantity of nitrogen. [b] Source of compounds: 1, prepared in this laboratory; 2, ICN Pharmaceuticals, Cleveland, Ohio; 3, Vega-Fox Biochemicals, Tucson, Ariz.; 4, Nutritional Biochemicals Corp., Cleveland, Ohio; and 5, Sigma Chemical Co., St. Louis, Mo. Data on commercial products were obtained from recrystallized materials. [c] Melting points (uncorrected) were determined on a Fisher–Johns melting-point block. [d] Optical rotation was determined on a Rudolph polarimeter (Rudolph Research, Fairfield, N.J.), model 80, sodium lamp, using 100-mm tubes (bore size of 3 mm) and a sample capacity of 0.7 ml, except where noted. [e] Elemental analyses were performed by the Microanalytical Laboratory, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. [f] *Cf.*, Ref. 10. [g] Optical rotation was measured in 50-mm tubes. [h] Crude.

The test solutions were prepared by adding an equivalent amount of 0.1 *N* NaOH to a prescribed amount of the test compound and diluting to volume, and they were sterilized by filtration through an all-glass system[1]. The pH of the assay system containing the maximum amount of test compound was checked on a duplicate set of tubes containing equivalent amounts of the ingredients and was within ±0.05 pH unit of the control tube containing all components except the test compound.

Inhibition studies were made initially in a system containing graded amounts of the test compound, *i.e.*, 0.1-, 0.5-, and 1.0-mg/ml final concentration; the highest concentration was the level specified in the microbiological antitumor screening protocol (12). When inhibition was noted in this system, the active compounds were compared on an equimolar basis at a final concentration of 4.47 μmoles/ml, which was equivalent to 1 mg of *N*-chloroacetyl-β-hydroxy-D-norleucine B[2]/ml, the compound found previously to be the most active in this system.

The organism used was *Lactobacillus casei* 7469[3], which was carried on agar[4], subcultured at least bimonthly, and transferred to a broth[4] for the preparation of the inoculum. The inoculum was a 1:20 suspension of the washed organism in normal saline. The riboflavin assay medium[4] was supplemented with the minimal amount of riboflavin[5] (0.03 μg/ml in the final system) required for optimal organism growth. The assay was incubated at 37° for 19 hr, after which growth was terminated by immersion of the assay tubes in boiling water for 10 min. The extent of growth was determined turbidimetrically on a Klett–Summerson colorimeter equipped with a red filter (660 nm). The details of the assay were described previously (1).

RESULTS AND DISCUSSION

The results of the inhibition studies of 27 benzoyl derivatives of amino acids and amino acid analogs containing graded amounts of the test compounds (up to 1 mg/ml final concentration) are shown in Table II. Of these, 19 showed some growth inhibition. The degree of inhibition ranged from a modest 13–33% for *N*-benzoyl derivatives of DL-allylglycine, β-hydroxy-DL-norleucine A and B, L-leucine, L- and DL-methionines, DL-norleucine, DL-norvaline, L-phenylalanine, L-tyrosine ethyl ester, and L-valine to a potent 89–96% inhibition for the benzoyl derivatives of *p*-chloro-DL-phenylalanine and *m*-fluoro-DL-phenylalanine. Intermediate inhibition was observed with other benzoyl derivatives, such as those of *o*-fluoro-DL-phenylalanine, *p*-fluoro-DL-phenylalanine, *p*-nitro-L-phenylalanine, β-2-thienyl-DL-alanine, β-3-thienyl-DL-alanine, and L-tryptophan.

The activity of the "modest" inhibitor observed in these experiments was comparable to that observed for most of the active chloroacetyl and trifluoroacetyl derivatives (2, 3). The "appreciable" inhibitory activity was considerably better than for most of the "active"compounds reported previously, being as active as *N*-chloroacetyl-β-hydroxy-D-norleucine B, the most active compound reported previously in this series (9). The activities of benzoyl-*p*-chloro-DL-phenylalanine and benzoyl-*m*-fluoro-DL-phenylalanine were the most pronounced yet observed based on the activity at 1 mg/ml, the concentration limit set in the protocol (12) to determine the positivity of the test compounds. Hence, five benzoyl compounds reported here (the benzoyl derivatives of *p*-chloro-DL-phenylalanine, *o*-fluoro-DL-phenylalanine, *m*-fluoro-DL-phenylalanine, *p*-nitro-L-phenylalanine, and L-tryptophan) meet the criterion of positivity. Further tests of these compounds in mammalian tumor systems are indicated.

The compounds showing little or no activity were not restricted to the benzoyl derivatives of nonessential amino acids since, in addition to the benzoyl derivatives of L-alanine, L-aspartic acid, L-glutamic acid, and glycine, those of L-arginine, L-histidine, L-lysine, and L-threonine also were inactive. With the exception of benzoyl-L-tyrosine ethyl ester, however, the active compounds were benzoyl derivatives of essential amino acids or of essential amino acid analogs.

When the 19 active compounds were compared on an equimolar basis (Table III), the more active ones were the benzoyl derivatives of phenylalanine analogs and the less active ones were derivatives of the alicyclic essential amino acid analogs. Benzoyl-L-phenylalanine and benzoyl-DL-norleucine were exceptions in that the former was somewhat less active and the latter was somewhat more active than other compounds of their respective groups. The degree of inhibition by the benzoyl alicyclic amino acids and analogs was approximately that noted for the chloroacetyl and trifluoroacetyl amino acids (2, 3). However, the degree of inhibition exhibited by the benzoyl derivatives of phenylalanine analogs was much greater than any inhibition noted previously with the *N*-acylated compounds. These results suggest that a number of other ring-substituted benzoyl derivatives should be tested, and such studies are now in progress.

Table II—Effect of N^{α}-Benzoyl Derivatives of Amino Acids and Amino Acid Analogs on Growth of *L. casei* 7469 [a]

N^{α}-Benzoyl Derivative	Concentration at 1 mg/ml, m*M*	Inhibition[b], % 0.1 mg/ml[c]	0.5 mg/ml[c]	1.0 mg/ml[c]
L-Alanine	5.12	0	2	8
DL-Allylglycine	4.56	2	8	15
L-Arginine (α-mono)	4.60	+3	+1	6
L-Aspartic acid	3.92	1	2	1
p-Chloro-DL-phenylalanine	3.29	6	60	89
o-Fluoro-DL-phenylalanine	3.48	5	21	50
m-Fluoro-DL-phenylalanine	3.48	7	84	96
p-Fluoro-DL-phenylalanine	3.48	5	23	46
L-Glutamic acid	3.84	+2	1	+5
Glycine	5.58	1	4	8
L-Histidine	3.61	+3	3	8
β-Hydroxy-DL-norleucine A	3.98	3	7	23
β-Hydroxy-DL-norleucine B	3.98	6	9	17
L-Leucine	4.25	2	13	26
L-Lysine (α-mono)	4.00	1	2	1
DL-Methionine	3.93	2	8	23
L-Methionine	3.93	2	10	16
p-Nitro-L-phenylalanine	3.18	4	28	56
DL-Norleucine	4.25	4	14	33
DL-Norvaline	4.52	2	7	14
L-Phenylalanine	3.59	2	14	29
β-2-Thienyl-DL-alanine	3.63	8	23	44
β-3-Thienyl-DL-alanine	3.52	6	22	41
L-Threonine	4.48	2	0	6
L-Tryptophan	3.15	5	28	53
L-Tyrosine ethyl ester	3.19	0	5	16
L-Valine	4.52	0	8	13
Benzoic acid[d]	8.19	3	6	9

[a] For details of assay, see Ref. 1. [b] Turbidity readings of the inoculated control tubes (containing no test compound) were 168–188 Klett units. At least three duplicate determinations were made for each compound. The duplicate values in each determination agreed within ±5 Klett units. The standard deviation of a compound showing a mean value of ~20% inhibition, for example, was 2.7 and the standard error was 0.95. The + indicates stimulation of growth but was considered insignificant. [c] Final concentration in assay system. [d] Free acid.

It is difficult to propose a mechanism of inhibitory action from these experiments. However, hydrolytic release of the constituent benzoic acid and the parent amino acid appears unlikely as a cause of inhibition in view of the findings that: (*a*) benzoic acid itself was innocuous in this system (Table II), (*b*) a mole equivalent of the free parent amino acid in this system under identical conditions exhibited less inhibition than the benzoyl compounds (2), (*c*) no free amino nitrogen was detectable in the test solution, indicating that no hydrolysis occurred during preparation of the solution, and (*d*) there was no detectable increase in the amount of amino nitrogen after the assay (2). Nevertheless, the possibility that the inhibition could be due to the membrane permeability of the benzoylated derivatives and an intracellular hydrolysis of the derivatives to free benzoic acid and the amino acid moiety is not excluded. Since not all benzoyl derivatives tested were inhibitory, intracellular hydrolysis seems an unlikely explanation unless the acylases involved exhibited specificity only to certain benzoyl amino acids.

Although the initial design of the test compounds was directed to an increase in lipophilic character, the present experiment, which was conducted solely to note inhibitory capacities, did not investigate the mechanism of action, and no attempt was made to correlate the lipophilic character with the inhibitory activity. A study of the mechanism of action requires a more sophisticated system and should include an investigation of such properties as relative lipid solubility, partition coefficients, and coacervation distribution values.

The similarity of inhibition shown by the *N*-benzoylated alicyclic amino acids with the *N*-chloroacetyl and *N*-trifluoroacetyl amino acids, which showed ~25% inhibition (2, 3), and the marked inhibition shown by the *N*-benzoylated phenylalanine analogs indicate that the mecha-

[1] Morton bacteriological filters, ultrafine porosity, Arthur H. Thomas Co., Philadelphia, Pa.

[2] The diastereomeric forms are arbitrarily referred to as Isomers A and B, the former moving faster and the latter slower when chromatographed on paper using methyl ethyl ketone–*n*-butanol–ammonia–water (3:5:1:1), ascending.

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

[4] Micro assay culture agar (Difco B319), micro inoculum broth (Difco B320), and riboflavin assay medium (Difco B325), Difco Laboratories, Detroit, Mich.

[5] Calbiochem, La Jolla, Calif.

Table III—Comparison of Effect of Equimolar Concentrations of *N*-Benzoyl Amino Acids and Amino Acid Analogs on Growth of *L. casei* 7469 [a]

N-Benzoyl Derivative	Inhibition [b], %
DL-Allylglycine	17
p-Chloro-DL-phenylalanine	94
o-Fluoro-DL-phenylalanine	82
m-Fluoro-DL-phenylalanine	97
p-Fluoro-DL-phenylalanine	72
β-Hydroxy-DL-norleucine A	14
β-Hydroxy-DL-norleucine B	22
L-Leucine	23
DL-Methionine	20
L-Methionine	18
p-Nitro-L-phenylalanine	78
DL-Norleucine	47
DL-Norvaline	13
L-Phenylalanine	39
β-2-Thienyl-DL-alanine	76
β-3-Thienyl-DL-alanine	56
L-Tryptophan	74
L-Tyrosine ethyl ester	26
L-Valine	16

[a] Maximum growth in inoculated control tube (containing no test compound) was 166–173 Klett units. For explanation of extent of variation of values, see footnote to Table II. [b] Concentration was 4.47 μmoles/ml and was the final concentration in the assay system. For details of assay, see Ref. 1.

nisms of inhibition of these compounds could be dissimilar. Certain tumor systems, as well as bacterial and mammalian asparaginase and glutamine synthetase, are inhibited by carbobenzoxy, phenacetyl, and phenylpropionyl amino acids (13–15).

Most compounds exhibiting notable inhibitory activity in the present studies were benzoyl derivatives of phenylalanine analogs. Therefore, it will be interesting to test the activity of these compounds against melanomas, wherein the phenylalanine metabolism is believed to be intimately involved. In view of the effect of similar compounds on mammalian tumor systems and certain isolated enzyme systems (13–15), the study of the activity of the compounds in asparaginase-sensitive tumors is also indicated.

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Degradation Kinetics of a New Cephalosporin Derivative in Aqueous Solution

ELISABETH S. RATTIE *, JAMES J. ZIMMERMAN ‡, and LOUIS J. RAVIN *x

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Abstract □ The degradation kinetics of a new cephalosporin derivative (I) in aqueous solution were investigated at 60°, $\mu = 0.5$, at pH 2.0–10.0. The observed degradation rates followed pseudo-first-order kinetics and were influenced significantly by H_2O and OH^- catalysis. No primary salt effect was observed in the acid region, but a positive salt effect was observed at pH 9.4. A general base catalytic effect by a phosphate buffer species was observed at pH 7–8. The pH–rate profile for I exhibited a degradation minimum at pH 6.05. The Arrhenius activation energies determined at pH 4.0 and 9.4 were 27.2 and 24.5 kcal/mole, respectively. Excellent agreement between the theoretical pH–rate profile and the experimental data supported the hypothesized degradation process. A comparison of I and cefazolin revealed close structural and stability analogies.

Keyphrases □ Cephalosporins—derivatives, degradation kinetics, aqueous solution, compared to cefazolin □ Antibacterial agents—cephalosporin derivatives, degradation kinetics, aqueous solution, compared to cefazolin □ Pharmacokinetics—cephalosporin derivatives, degradation, aqueous solutions, compared to cefazolin

Yamana and Tsuji (1) reported the comparative stability of several commercially available cephalosporin derivatives including those with an α-amino group in their side chain (*e.g.*, cephalexin, cephradine, and cephaloglycin) and those lacking the side-chain α-amino group (*e.g.*, cephalothin, cephaloridine, and cefazolin). Cephalosporins possessing the α-amino group may be administered orally, and cephalexin and cephradine have the highest stability of the derivatives investigated. Cephalosporins lacking the α-amino group are not administered orally, and cefazolin in particular is very unstable in the acid pH region.

This report describes the stability kinetics of a new cephalosporin derivative, 3-[2-(5-methyl-1,3,4-thiadiazyl)mercaptomethyl]-7-[D-(−)-mandelamido]-3-cephem-4-carboxylic acid sodium salt (I) (Table I). Of the cephalosporin derivatives previously investigated (1), I most closely resembles cefazolin. These two compounds have the same R_2 group but different R_1 groups. The continued investigation of cefazolin-like derivatives appears to be warranted in view of the clinical advantages of cefazolin over other cephalosporins (2).

BACKGROUND

As a result of previous studies (1, 3), the following conclusions may be

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