Synthesis of some valine derivatives as potential antibacterial agents

Frederick Nelson Minard
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UMI®
SYNTHESIS OF SOME VALINE DERIVATIVES
AS POTENTIAL ANTIBACTERIAL AGENTS
by
Frederick N. Minard

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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In Charge of Major Work

Head of Major Department

Dean of Graduate College

Iowa State College

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INTRODUCTION

Among the features of many peptide-like antibiotics are specific structures and the occurrence of certain amino acids in the atypical form. Because the field of antibiotics is recent in development, there has been very little report of research which is directed toward the correlation of antibacterial activity with structure and optical isomerism. Undoubtedly there will be a trend away from the isolation of antibiotics and toward the preparation of materials similar to them, but only after their structural characteristics have become more known and evaluated. The present thesis involves the synthesis of acyl derivatives of valine which may possibly have antibacterial action. The acyl groups have been chosen because they offer a wide range of structures, and many of them can occur as geometrical isomers. The three optical forms of valine have been incorporated into some of the derivatives. Valine was selected since it is fairly common in antibiotics, sometimes occurring as the $L$ form and sometimes as the $D$ form.

There are no clearly defined principles enabling one to predict structures which can give antibacterial activity to amino acid derivatives. In other investigations of substances exhibiting a biological effect, success has often been obtained by the screening of many types of structures.
A bacteriological investigation of the compounds prepared for this thesis may offer a guide to such principles.
A useful definition of an antibiotic is that given by Waksman (1): An antibiotic is "a chemical substance produced by microorganisms which has the capacity to inhibit the growth of bacteria and other microorganisms and even to destroy them."

Inhibition may be considered as a retardation or cessation of enzymic processes within a microorganism caused by an influence usually foreign to that organism and evidenced by a lack of growth or multiplication.

Waksman (1) has defined antagonism as

the phenomenon of a living organism inhibiting or interfering with the activities of another living organism as a result of the creation of unfavorable conditions in the medium or the production of a specific antimicrobial substance.

The atypical configuration of an amino acid is the antipode of that isomer which usually occurs in nature. Since this antipode sometimes does occur naturally, the often-used term "unnatural configuration" is not properly descriptive.

HISTORICAL

The Structure of Peptide-like Antibiotics

It is believed that Pasteur and Joubert (2) were the first to call attention to bacterial antagonism and to suggest its use against infections. Since then, but only after a long lapse of time, there has been developed a large background of chemical and microbiological information concerning antagonism and the metabolic products which cause it. These products have been termed "antibiotics" by Waksman (3). In 1899 Emmerich and Leow (4) reported the antibacterial activity of the culture medium from Pseudomonas pyocyaneus (Pseudomonas aeruginosa) as being due to a substance "pyocyanase." This was the first antibiotic discovered. Subsequent reports of antibiotic materials were the following: penicillic acid in 1913 (5), actinomycetin in 1924 (6), penicillin in 1929 (7), citrinin in 1931 (8), and gliotoxin in 1936 (9).

A surge of interest in the antibacterial effects of these metabolic products began in 1939 when Dubos and

(2) Pasteur and Joubert, Compt. rend., 85, 101 (1877).
(9) Weindling and Emerson, Phytopathology, 26, 1068 (1936).
Gattaneo (10) reported the strong activity of tyrothricin, a protein fraction isolated from cultures of *Bacillus brevis*. Since then numerous other antibiotics have been isolated and investigated both chemically and bacteriologically. This review will consider only those antibiotics which have been shown to contain or can possibly be derived from amino acids, and whose structure may contribute something to the problem. With several possible exceptions the complete structures of these antibiotics are unknown. This lack of information is perhaps due to a variety of reasons, of which the following are important: the molecules are often very complex in character, protein chemistry has not advanced to the position where the arrangement of amino acids is easily determined, and finally, the field of antibiotics is relatively new in development.

Much interest has been attached to the fact that many of these antibiotics contain amino acids in the atypical configuration. Those which have been reported are the following: gramicidin (D-leucine) (11,12) and (D-valine) (13,14), tyrocidine (D-phenylalanine) (15), gramicidin S (D-phenylalanine) (16),

(12) Hotchkiss, *ibid.*, 141, 171 (1941).
(14) Hotchkiss, *J. Bact.*, 45, 64 (1944).
penicillin (D-penicillamine) (17), bacitracin, (D-phenylalanine) (18), and aerosporin (D-leucine and D-\(\alpha\), 7-diaminobutyric acid) (19).

Various hypotheses concerning the function of the D residues in these molecules have been presented. From the evidence available it appears that if this particular configuration is critical for activity, it may be but one of several critical features. For example, the inclusion of the D configuration in simple amino acid derivatives does not produce any antibacterial activity which the corresponding L derivative does not possess (20, 21, 22). Perhaps of fundamental importance is the fact that the presence of D-amino acids as such (23) and incorporated into simple peptides (24) interferes with the action of proteolytic enzymes. In this connection Hotchkiss stated (25),

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(24) Stahmann, Fruton, and Bergmann, Ibid., 164, 753 (1946).
These $D$-amino acids may be toxic when combined, or the peptide containing them may "compete" with some normal protein or peptide. However, it is more likely that the unnatural amino acids contribute rather a measure of biological indestructibility to a molecule toxic for other reasons.

The inhibition of bacterial growth by $D$-amino acids under laboratory conditions (20, 26, 27, 28) strengthens the idea that the inclusion of this configuration in antibiotics may contribute to the antibiotic action.

Miss Crowfoot has briefly indicated (29) that the $D$ configuration of the phenylalanine in gramicidin S may facilitate cyclization. This idea has been further elaborated by Neuberger (30), who suggested that the presence of the $D$ residues in a cyclic polypeptide reduces the steric interference between the protruding side chains. It may thus be considered that a cyclic polypeptide composed of amino acid residues all of which have the same configuration would tend to be more unstable than a similar cyclic polypeptide containing a proper proportion of both configurations.

* The assistance which the inclusion of glycine may give to the cyclization of a polypeptide should be considered. Neurath, J. Am. Chem. Soc., 65, 2039 (1943).

Concerning the effect of the inclusion of the D configuration Neuberger (31) stated,

On this basis the cyclic character and the configurational abnormalities are related, and the biological properties of these compounds may be due, not so much to D configuration as such, but to the cyclic structure which affects solubility and other physical properties and thus largely determines the penetration of these substances into cells.

A protein precipitate, obtained at a pH of 4.5 from cultures of Bacillus brevis, was treated to obtain an alcohol-soluble water-insoluble fraction (10), which was subsequently named "tyrothricin" (32). Dubos and Hotchkiss (33,34) showed that its antibacterial activity was due to the presence of two substances, gramicidin and tyrocidine, and they obtained both of these antibiotics in crystalline form, the latter occurring as a hydrochloride. It has been estimated (35) that tyrothricin contains 10-20% of gramicidin and 40-60% of tyrocidine hydrochloride. Both antibiotics are resistant to the proteolytic enzymes, trypsin, pepsin, papain, and papaya latex (12). This has been accounted for by the presence of D-amino acids and the high content of hydrophobic side chains (35).

(33) Hotchkiss and Dubos, Ibid., 132, 791, 793 (1940).
(34) Hotchkiss and Dubos, Ibid., 141, 155 (1941).
Gramicidin has been considered to be a cyclic poly-peptide composed of twenty-two amino acid residues and two ethanolamine residues (35). These could be combined to form twenty-four -CO-NH-linkages. The antibiotic contains free hydroxyl groups but no free carboxyl or amino groups (36). Hence it is neutral in aqueous solution. Gordon, Martin, and Synge (37) have determined by partition chromatography that the amino acid composition of gramicidin, using twenty-four residues as a molecular unit, is as follows:

<table>
<thead>
<tr>
<th>Residues</th>
<th>No. of Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-alanine</td>
<td>3</td>
</tr>
<tr>
<td>glycine</td>
<td>2</td>
</tr>
<tr>
<td>D-leucine</td>
<td>6</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>6</td>
</tr>
<tr>
<td>D- and L-valine</td>
<td>5</td>
</tr>
<tr>
<td>hydroxyamino compound*</td>
<td>2</td>
</tr>
</tbody>
</table>

D-Leucine (11,12) and DL-valine (13,14) have been isolated from an acid hydrolysis mixture. The presence of D-amino acids had been previously indicated by the action of a D-amino acid oxidase upon the hydrolysate (38). By comparison of the ultraviolet spectra of acetyl tryptophan and gramicidin, Edwards (39) estimated the tryptophan content of gramicidin to be 39.3%, as compared with 38.1% for a

(38) Lipmann, Hotchkiss and Dubos, J. Biol. Chem., 141, 163 (1941).
chemical determination (40). This has been stated to be about five times the tryptophan content of any known protein (41).

By counter-current distribution Gregory and Craig (42) separated crystalline gramicidin into two main components, gramicidin A with a melting point of 227-228° and gramicidin B with a melting point of 258-259°, and at least two smaller components. There was also a very small band apparently corresponding to tyrosine. Each component gave spectral evidence for tryptophan; gramicidin A had the highest percentage and gramicidin B had about 85% that of A. Paper chromatography indicated that both gramicidin A and B contained alanine, glycine, leucine, tryptophan, and valine.

Because the above evidence shows that crystalline gramicidin is nonhomogeneous, previous hypotheses concerning structure and molecular weight must be revised. The presence of these components may well explain the lack of agreement among the various molecular weight determinations*

Not much is known about the arrangement of residues in gramicidin. After strong acid hydrolysis Christensen (43)

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* For summary, see (35).

(42) Gregory and Craig, ibid., 172, 839 (1948).
isolated the dipeptides $D$-valyl-$D$-valine and $L$-valyl-$L$-valine as a racemic mixture. There has been some discussion as to whether these configurations occur related as such in gramicidin (30, 44). However, at present there are not enough data available on the relationship between peptide structure and ease of inversion to determine whether inversion at these optical centers could, or could not, have occurred during hydrolysis and isolation (30).

Synge (44) has isolated the dipeptide $L$-valylglycine from a partial hydrolyzate of gramicidin. From the amount of the dipeptide obtained, he stated that in the antibiotic at least two $L$-valine residues occur linked through their carboxyl groups to glycine residues.

The structure of tyrocidine has been clarified no more than that of gramicidin. It is known to be a polypeptide having a weakly acidic group, two basic groups and three ammonia residues occurring as amides (12). Gordon, Martin, and Synge, using partition chromatography on an acid hydrolyzate (15), have isolated and identified as acetyl derivatives $L$-aspartic acid, $L$-glutamic acid, $L$-leucine, $L$-ornithine, $D$-phenylalanine, $L$-proline, tryptophan (rotation not determined), $L$-tyrosine, and $L$-valine. The presence of

\[ L\text{-aspartic acid} \] was confirmed by the isolation of its benzoyl derivative from a hydrolysate (45); that of \[ L\text{-leucine}, \]
\[ L\text{-valine}, \] and \[ L\text{-tryptophan} \] was confirmed by microbiological assay (45). The following composition (35) takes into consideration the analytical data now available although a molecule about one-half the size has been suggested as also satisfying the data (46):

<table>
<thead>
<tr>
<th>Residue</th>
<th>No. of Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>ammonia</td>
<td>3</td>
</tr>
<tr>
<td>( \text{L-aspartic acid} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-glutamic acid} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-leucine} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-ornithine} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-phenylalanine} )</td>
<td>3</td>
</tr>
<tr>
<td>( \text{L-proline} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-tryptophan} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-tyrosine} )</td>
<td>2</td>
</tr>
<tr>
<td>( \text{L-valine} )</td>
<td>2</td>
</tr>
</tbody>
</table>

By splitting out twenty-two equivalents of water, the above molecules could condense to a large molecule whose hydrochloride would have the empirical formula \( \text{C}_{127}\text{H}_{166}\text{N}_{26}\text{O}_{26}\cdot 2\text{HCl} \), and a molecular weight of 2546 (35). Hotchkiss has reported (12) equivalent weights of 855 by alkali titration, 1285 by chloride determination and 815 by the determination of easily evolved ammonia. His hypothesis for the structure of tyrocidine is of interest (47):

A likely arrangement of the residues is a peptide chain in which the 19 \( \alpha \)-amino and

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(46) Christensen, ibid., 160, 75 (1945).
(47) Hotchkiss, Advances in Enzymol., 4, 168 (1944).
carboxyl groups are combined, presumably formed in a cycle. The 2 basic groups of the ornithine side chains would be free to combine as hydrochloride, while 3 of the 4 acidic side chains of the dicarboxylic acids are probably combined with ammonia as -CO-NH₃⁺ groups. The remaining free carboxylic side chain presumably accounts for the free weak acid group found when tyrocidine is titrated by alkali in alcoholic solution. Of course, it is also possible that this group is combined and one of the α-amino acid carboxyl groups is free. It had been suggested that the free weak acid group was a phenolic group of tyrosine because the acetyl derivative appeared to be neutral (12), but this observation may have been in error.

Christensen (46) has reported experiments in which he showed that the acidity of tyrocidine is due to the phenolic hydroxyl of tyrosine and that the only free basic groups are due to the 8-amino group of ornithine. Treatment of tyrocidine with p-toluenesulfonyl chloride and subsequent acid hydrolysis gave only the corresponding tosyl ester of 3-tyrosine and the 3-tosylamide of 3-ornithine. Methylation of tyrocidine with dimethyl sulfate gave the methyl ether of 3-tyrosine; reaction of tyrocidine with methyl isothiourea produced 3-arginine. He detected no other amino acid derivatives in the reaction mixtures following hydrolysis.

Gramicidin S (Soviet gramicidin) (48,49) is a crystalline polypeptide hydrochloride which is isolated from a

---

(49) Belozersky and Passhina, Ibid., 247, 716 (1944).
variety of *Bacillus brevis*. Its structure has been definitely determined.

Synge (16) suggested that the substance is cyclic in structure because of the following facts: the presence of one free amino group per stoichiometric minimum unit, the absence of free carboxyl groups, and a very large optical rotation, which is \([\alpha]_D^{20} = -295^\circ\) in 70% ethanol. From the results of paper chromatography on an acid hydrolyzate and subsequent isolation of acetyl amino acids (16), Synge showed that this minimum unit of gramicidin S is composed of one residue of each of the following amino acids*: \(L\)-leucine, \(L\)-ornithine, \(D\)-phenylalanine, \(L\)-proline, and \(L\)-valine. By the use of 2,4-dinitrofluorobenzene Sanger (50) demonstrated that the basic character is due to the \(D\)-amino group of \(L\)-ornithine.

By paper chromatography of a partial hydrolyzate, Consden, Gordon, Martin, and Synge (51) obtained enough evidence to determine the complete structure of gramicidin S. The following peptides were isolated: \(\alpha\)-valylornithine, ornithyleucine, leucylphenylalanine, phenylalanlylproline, and also though less conclusively, \(\alpha\)-prolylvalylornithine, \(\alpha\)-valylornithyleucine, and phenylalanylprolylvaline.

* The similarity in amino acid composition between gramicidin S and tyrocidine should be noted.

Because of the overlapping structure of these peptides, they were able to formulate unequivocally the arrangement of residues in gramicidin S as \((\cdots(\text{L-valyl-L-ornithyl-L-leucyl-L-phenylalanyl-L-prolyl-})\cdots)^n\), where \(n\) is probably 1 or 2.

It has been pointed out (16) that the knowledge concerning gramicidin S will likely prove helpful in the structure determination of tyrocidine, which is similar in amino acid composition and in which the \(\delta\)-amino group of ornithine is also free.

The free \(\delta\)-amino group of ornithine in gramicidin S was shown to be a critical feature for antibacterial activity by Znamenskaya, Agatov, and Belozerskii (52). All activity was lost by its acetylation, benzoylation, or deamination with nitrous acid. Salts with nucleic acids retained their activity.

The antibiotic activity of penicillin was first noted by Fleming (7) in a bacterial culture contaminated by *Penicillium notatum*. In 1940, a group of workers (53) re-investigated the activity and thereby initiated an enormous


program of chemical research (54), as well as much bacteriological and clinical research.

The structure of the penicillin molecule was established by a variety of chemical and physical methods (54). It was shown to consist of a β-lactam ring fused to a thiazolidine ring. The sulfur-containing amino acid, penicillamine, has the α-configuration (55).

A number of naturally occurring penicillins have been isolated in which the R group is varied (56). They are the following: n-amylpenicillin, 2-pentenylpenicillin, n-heptylpenicillin, benzylpenicillin, and p-hydroxybenzylpenicillin.

Several parts of the penicillin molecule have been shown to be critical for antibiotic activity. The α-configuration of penicillamine is probably essential; this fact has been shown by indirect evidence (57). The β-lactam

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(55) Abraham, Baker, Boon, Calam, Carrington, Chain, Florey, Freeman, Robinson, and Sanders, Ibid., p. 15.
ring is essential; hydrolysis to the di-acid removes the activity (58), and a homologue of penicillin with a \(\gamma\)-lactam ring is inactive (59). The oxidation of the sulfide group to a sulfoxide (60, 61) produces inactivity. The oxidation to a sulfone (61) or the substitution of the two methyl groups by hydrogen (62) does not completely remove the antibacterial activity.

Crystalline benzylpenicillin has been synthesized (57, 63) in a very low yield (0.1%) from \(\beta\)-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone. However, the synthesis was of such a character that it cannot be considered as a confirmation of structure.

Bacitracin is a polypeptide obtained from a strain of *Bacillus subtilis* (64). It resists digestion by pepsin or trypsin (64).

A commercial sample of bacitracin was separated by counter-current distribution into one main band (83% of the sample weight) and two smaller bands (18). After acid hydrolysis of this main band there were isolated, again by counter-current distribution, these five bands: a

---

(59) du Vigneaud and Carpenter, ibid., p. 1004.
(60) Reck and Folkers, ibid., p. 144.
(61) Cognill, Stodola, and Wachtel, ibid., p. 680.
(62) du Vigneaud, Wood, and Wright, ibid., p. 892.
(63) du Vigneaud, Carpenter, Holley, Livermore, and Rachele, ibid., p. 1018.
(64) Johnson, Anker, and Heleney, *Science*, 102, 376 (1945).
crystalline dipeptide with little or no optical activity but containing phenylalanine and isoleucine, a peptide apparently containing phenylalanine and ornithine, racemic phenylalanine, L-leucine, and finally, L-isoleucine.

Moore and Stein (65) reported the preliminary analysis of bacitracin by the aid of a starch column. They obtained the following data in terms of grams of amino acid per one hundred grams of bacitracin:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>1.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>17</td>
</tr>
<tr>
<td>Cystine</td>
<td>14</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>10</td>
</tr>
<tr>
<td>Histidine</td>
<td>10</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>22</td>
</tr>
<tr>
<td>Leucine</td>
<td>9</td>
</tr>
<tr>
<td>Lysine</td>
<td>7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>11</td>
</tr>
</tbody>
</table>

An undetermined amino acid, perhaps ornithine or hydroxyproline, moved down the column with cystine. The authors stated that within the sensitivity of the method (0.1%) the following amino acids were absent: arginine, methionine, proline, serine, threonine, and valine.

Aerosporin (66) is produced by the organism *Bacillus aeroperous* Greer. By paper chromatography of an acid hydrolyzate (19), Jones demonstrated the presence of leucine, threonine, and α,γ-diaminobutyric acid. The latter was

(65) Moore and Stein in (18).
believed to be in the DL form because of the similarity of the melting point of its hydrochloride to that of a synthetic sample. After spraying the chromatogram with D-amino oxidase and incubation (67), treatment with ninhydrin revealed that the isomers were D-leucine and L-threonine. No confirmation of the configuration of the di-amino acid could be obtained from this experiment because a synthetic sample of D-ωγ-diaminobutyric acid was not affected by the D-amino acid oxidase used.

Polymyxin, produced by *Bacillus polymyxa*, is unusual among antibiotics in that it is specific for Gram-negative bacteria (68, 69). It is the hydrochloride of a basic polypeptide and contains no detectable acidic groups (70). Polymyxin, like some other antibiotics, e.g., gramicidin and tyrocidine, is resistant to hydrolysis by enzymes. Pepsin, trypsin, pancreatin, and erepsin have been tried (71).

Chloromycetin (72), produced by a variety of *Streptomyces*, may become of importance because of its activity.
against experimental rickettsial and viral infections (73).

\[
\text{NO}_2^- \quad \text{O} \quad \text{CH}_2\text{OH} \quad \text{CH}_2\text{OH}
\]

The structure (74) is extremely unusual among natural products because of the presence of a nitro group and a di-chloroacetyl radical. It may be considered as an amino acid derivative in that phenylalanine could conceivably be the precursor.

Aspergillic acid (75) is produced by *Aspergillus flavus*. It is too toxic for clinical use (75). The structure resembles the anhydride of isoleucine (76) and a notable feature of it is the presence of a hydroxamic acid group. This group has been demonstrated to be the center of antibiotic activity since the desoxy derivative is inactive and several synthetic hydroxamic acids also possess antibacterial activity (76).

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(74) Hebstock, Crooks, Controule, and Bartz, Paper at 115th meeting, American Chemical Society, San Francisco, California. 1949.
A number of articles have appeared which report experiments designed to produce synthetic amino acid derivatives having antibacterial action. For the purpose of this review they may be considered in two groups: modified antibiotics and purely synthetic compounds containing a natural amino acid. The latter compounds have usually been modeled after a characteristic of some particular antibiotic.

A property of gramicidin which deters its use in medicine is its strong hemolytic action. In an attempt to reduce hemolysis and also to obtain a more soluble substance, a group of workers (40, 77, 78) treated gramicidin with a number of reagents which are capable of reacting with an indole nucleus or with an hydroxy 1 group. The action of formaldehyde, which introduced methylol groups into the indole nucleus, decreased the amount of hemolysis to 20% and the antibacterial activity to 50% that of gramicidin. The solubility was not increased. The reaction product with glyoxylic acid retained the hemolytic action and this was not reduced by subsequent treatment with formaldehyde. The sulfamic acid obtained by the reaction of pyridine-chlorosulfonic acid showed both decreased hemolytic and antibacterial

action. A succinic half ester of methylol-gramicidin showed about 24% of the antibacterial activity, 1-5% of the hemolytic activity and about 2-5% of the toxicity of gramicidin. Its salts were soluble in water.

Other derivatives of gramicidin were prepared by Schales and Mann (79, 80). Their formaldehyde reaction product had about 15% the hemolytic activity of gramicidin. The substances they obtained by treatment of gramicidin with nitrous acid, chromic acid, bromine, iodine, hydroxylamine, hydrogen chloride, and sodium hydroxide were 2-87% less hemolytic and 18-50% less active against Staphylococcus aureus than the original material.

Many modified penicillins have been prepared in an attempt to improve their effectiveness (60, 61), either by increasing their stability, decreasing their rate of excretion, or changing their specificity to organisms. The benzyl and alkyl esters proved to be inactive in man because of the esters' resistance to hydrolysis. The \( \beta \)-diethylamino ester of benzylpenicillin is readily hydrolyzed at \( \text{pH} \) 6.0 (81), but its activity has not been reported. \( \epsilon \)-Hydroxybenzylpenicillin was iodinated to a diiodopenicillin and

(79) Schales and Mann, Arch. Biochem., 13, 357 (1947).
(80) Schales and Mann, Ibid., 18, 217 (1948).
brominated to an unpurified bromopenicillin. Each product was slightly more active in vitro than the parent material. A group of azo penicillins was obtained by the coupling of aryl diazonium salts with p-hydroxybenzylpenicillin. In general, they proved to have the same order of in vitro activity as the parent penicillin. The sulfone of benzylpenicillin had appreciable in vitro and in vivo activity. The corresponding sulfoxide was reported to be inactive in vitro. Nitroration of benzylpenicillin gave a product which was also inactive in vitro.

Some new penicillins have been produced by the addition of suitable compounds containing a substituted acetyl radical to the growth media (82, 82). The mold proved able to incorporate certain of these radicals into a penicillin molecule. Twenty-nine crystalline penicillins have been obtained in this manner. They are p-methoxybenzyl-, p-nitrobenzyl-, p-, m-, and p-fluorobenzyl-, p-chlorobenzyl-, p-bromobenzyl-, p-iodobenzyl-, 2-thiophenemethyl-, phenoxyethyl-, p-tolylmercaptomethyl-, cyclopentylmethyl-, p-methylbenzyl-, p-allyloxybenzyl-, methylmercaptomethyl-, ethylmercaptomethyl-, p-propylmercaptomethyl-, isopropylmercaptomethyl-, allylmercaptomethyl-, p-bromoallylmercaptomethyl-

(83) Behrens, Corse, Edwards, Garrison, Jones, Soper, Van Abeele, and Whitehead, J. Biol. Chem., 175, 793 (1948).
n-butylmercaptomethyl-, isoamylmercaptomethyl-

m-trifluoromethylphenylmercaptomethyl-, \( \gamma \)-phenylpropylmer-
captomethyl-, \( \beta \)-phenoxyethylmercaptomethyl-

\( \beta \)-naphthylmercaptomethyl-, phenylselenomethyl-
p-methoxyphenoxyethyl-, and 3-thiophenemercaptomethyl-

penicillin.

No synthetic derivative of \( \text{D} \) amino acids has yet been
reported which has antibacterial activity approaching that
of the antibiotics. The evidence points to the fact that
the mere inclusion of a \( \text{D} \) configuration into an amino acid
derivative is not enough to confer antibiotic activity.

During his research on gramicidin and tyrocidine, Hotch-
kiss (14) tested palmityl-\( \text{L} \)- and \( \text{DL} \)-tryptophan. Both iso-
mers showed about the same activity. Fling has reported the
testing of the following compounds (20): \( \text{D} \)- and \( \text{L} \)-leucine
methyl ester, \( \text{D} \)- and \( \text{L} \)-valine methyl ester, \( \text{D} \)- and \( \text{L} \)-tyrosine
ethyl ester, \( \text{N} \)-benzoyl-\( \text{D} \)- and \( \text{L} \)-tyrosine, \( \text{N} \)-benzoyl-\( \text{D} \)- and
\( \text{L} \)-tyrosine amide and ethyl ester, \( \text{DL} \)-leucine anhydride, the
formyl, glycy1, phthaloy1, \( \text{DL} \)-proly1 derivatives of \( \text{D} \)- and
\( \text{L} \)-leucine, and the formyl, phthaloy1, \( \text{DL} \)-proly1 derivatives
of \( \text{D} \)- and \( \text{L} \)-valine. They were all relatively inactive and
showed no antipodal specificity.

The four isomeric leucylleucines, \( \text{L}-\text{L} \)-, \( \text{D}-\text{L} \)-, \( \text{L}-\text{D} \)-, and
\( \text{D}-\text{D} \)-, were tested (22) and found to be devoid of antibacterial
activity. The "racemization" of casein gave materials which also had no activity (22).

The synthesis of D-leucyl-L-tryptophan anhydride has been reported by Pruton (84). He thought it of interest because gramicidin is considered to be a cyclic polypeptide containing a large proportion of both D-leucine and L-tryptophan. The compound was but slightly active against the organisms used.

Because degradation experiments on gramicidin S (51) showed the structure to be (\(-\alpha-(L-valyl-D-ornithyl-D-leucyl-D-phenylalanyl-L-prolyl-)\))\(_n\), Harris and Work (85) prepared these two peptides: \(\alpha-L-valyl-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline\) and \(\alpha-L-valyl-DL-ornithyl-L-leucyl-L-phenylalanyl-L-proline\). DL-ornithine was used because of its greater availability as compared with L-ornithine. Both peptides were only very slightly inhibitory against certain organisms and showed no detectable antipodal specificity. The authors stated that the corresponding peptides containing L-ornithine would be prepared and that attempts would be made to cyclize them.

In connection with the structure determination of gramicidin S, Synge (86) prepared these dipeptides:

α-L-valyl-L-ornithine, L-ornithyl-L-leucine, L-leucyl-D-phenylalanine, D-phenylalanyl-L-proline and L-prolyl-L-valine. There were no antibacterial tests reported.

Typical of the substances which a group of workers has prepared (87, 88, 89), using penicillin as a model, is the following structure:

\[ R^*-\text{CO-NH-CHR-CO-H} \]

where \( R \) represents various amino acid side chains, \( R^* \) is hydrogen or methyl, and \( R^\prime \) is phenyl or benzyl. They also prepared simpler derivatives having this general structure:

\[ R^\prime \text{-CO-NH-CHR-CO-N}^\prime \text{-CO}_2\text{H} \]

where \( R \) is of a type such as trichloromethyl or isopropyl, and \( R^\prime \) is hydrogen or methyl. In the latter compounds cysteine or penicillamine was incorporated as the DL, D, and L forms. None of the derivatives showed much antibacterial activity. Syntheses of other amino acid derivatives having a structure similar to that in the latter

figure given above have been reported (90, 91).

In a patent (92) there has been described the synthesis of this compound:

\[
\text{G}_{6}\text{H}_{5}\text{-CS-} \text{NH-CH}_{2}\text{-CO-NH-CH}_{3}\text{CH}_{3}\text{CO}_{2}\text{H}
\]

It had no antibiotic activity but after treating it with silver oxide in chloroform, there was isolated a material having "penicillin-type" activity.

(90) British Patent 584, 918 (1947).
EXPERIMENTAL*

Preparations Related to the Problem

**Phthaloyl-DL-valine**

This compound was prepared by the fusion of 14.8 g. (0.100 mole) of phthalic anhydride with 11.7 g. (0.100 mole) of DL-valine at 175° for about twenty minutes and until all water was removed. The residue was recrystallized from 100 ml. of carbon tetrachloride to give 24 g. (97% of theory) of product with a melting point of 99-101°. Upon repeated recrystallization of this material from cyclohexane, crystals having a melting point of 102-103° were obtained.

Anal. Calcd. for C_{13}H_{13}O_4N: neut. equiv., 247; N, 5.67.

Found: neut. equiv., 246; N, 5.67.

**Phthaloyl-DL-valine monohydrate**

Three g. of the above imide was dissolved in 6 ml. of absolute ethanol and enough water was added to cause turbidity. Upon careful cooling, 2.3 g. of the monohydrate

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* Melting points are uncorrected. Rotations, unless otherwise noted, were taken on 3% solutions in absolute ethanol. Nitrogen determinations were done by the micro Kjeldahl method.

** Derivatives marked with a # have been reported, Minard and Fox, J. Am. Chem. Soc., 71, 1160 (1949).

*** This derivative had been prepared prior to its description by Billman and Harting, J. Am. Chem. Soc., 70, 1473 (1948).
crystallized out with a melting point of 80.0-81.5° after preliminary softening.

Anal. Calcd. for C_{13}H_{15}O_5N: neut. equiv., 265;
N, 5.28.

Found: neut. equiv., 262; N, 5.28, 5.35.

Phthaloyl-\(\beta\)- and \(L\)-valine

These derivatives were synthesized by fusion of phthalic anhydride and the valine isomer as described above for the phthaloyl-\(\beta L\)-valine. They have been previously reported (20,21).

\(\textit{cis}-1,2\)-Cyclobutanedicarboxylic anhydride

The \(\textit{cis}-1,2\)-cyclobutanedicarboxylic anhydride was prepared according to the method in the literature (93) through the following sequence of intermediates: adipic acid, \(\alpha,\alpha\prime\)-dibromo-adipic acid dimethyl ester, \(1\)-cyano-1,2-cyclobutanedicarboxylic acid dimethyl ester, \(1,1,2\)-cyclobutanetricarboxylic acid, \(\textit{cis}-1,2\)-cyclobutanedicarboxylic acid, and finally, \(\textit{cis}-1,2\)-cyclobutanedicarboxylic anhydride.

One and three-tenths g. (0.010 mole) of the anhydride and 1.2 g. (0.010 mole) of \(\beta L\)-valine were fused in an oil-

bath at 175° for about twenty minutes, and the residue was recrystallized once from carbon tetrachloride to give 1.0 g. of material, melting point 99-100°. Subsequent recrystallizations from cyclohexane gave crystals with a melting point of 102.5-103.5°.

Anal. Calcd. for C_{11}H_{15}O_{4}N: neut. equiv., 225; N, 6.22.
Found: neut. equiv., 223; N, 6.18, 6.20.

cis-1,2-cyclobutanedicarbonyl-DL-valine monohydrate

When the above imide was dissolved in water at 60° and the solution cooled, crystals of the monohydrate appeared. The melting point could not be raised above 80-83° by purification from water.

Anal. Calcd. for C_{11}H_{17}O_{5}N: neut. equiv., 243; N, 5.76.
Found: neut. equiv., 241; N, 5.74.

cis-1,2-cyclobutanedicarbonyl-D-valine monohydrate

The fusion between 2.3 g. (0.020 mole) of the anhydride and 2.5 g. (0.020 mole) of D-valine was carried out at 175° in the usual manner. This isomer was purified from ethanol-water to give crystals of a monohydrate whose melting point remained at 92-98°. The anhydrous compound, from tetralin-hexane, was very hygroscopic and was not investigated.

\[ \xi / D = +77.5° + 3.6° \]
Anal. Calcd. for C_{11}H_{17}O_5N: neut. equiv., 243; N, 5.76.

Found: neut. equiv., 246; N, 5.81, 5.85

\[ \text{\textsuperscript{cis}-1,2-Cyclobutanedicarbonyl-L-valine monohydrate} \]

The preparation and the quantities used were in every way identical with those described for the \( \text{D} \) isomer, \( \text{L} \)-valine being used instead of the \( \text{D} \)-valine. The melting point was also 92-98\(^\circ\).

\[ \left( \alpha \right)_D = -76.1^\circ \pm 2.8^\circ \]

Anal. Calcd. for C_{11}H_{17}O_5N: neut. equiv., 243; N, 5.76.

Found: neut. equiv., 246; N, 5.70, 5.73.

\textbf{Procedure for the hydrolysis of the imides}

A general procedure was used for the hydrolysis of each of the preceding imides: A quantity of the imide (1-12 g.) was titrated with 2 \( \text{N} \) sodium hydroxide to a phenolphthalein end point, and the identical amount of base plus 20\% of the total was added. The resulting solution was placed in a vigorously boiling water-bath and heated until the inside temperature reached 70\(^\circ\). It was then quickly cooled in ice and acidified with excess concentrated hydrochloric acid. After precipitating in the cold, the solid, consisting of organic and inorganic material, was filtered off and purified as described below for each compound. The yields of purified
2-carboxycyclobutanecarbonylvalines and \( \alpha \)-carboxybenzoyl-
valines were 40-50\% and 60-75\% respectively. The substances
were all more water-soluble than the imides from which they
were prepared.

\#o-Carboxybenzoyl-\( \text{DL} \)-valine

The dried material from hydrolysis was dissolved in
acetone, filtered from the organic residue, and precipitated
by carbon tetrachloride. Several such purifications gave
crystals with a melting point of 171.5-172.0\°.

Anal. Calcd. for \( \text{C}_{13} \text{H}_{15} \text{O}_{5} \text{N} \): neut. equiv., 133; N, 5.28.

Found: neut. equiv., 132; N, 5.27, 5.27.

\#o-Carboxybenzoyl-\( \text{L} \)-valine

This derivative was obtained from phthaloyl-\( \text{L} \)-valine
and was purified in the same manner as described above for
the \( \text{DL} \) isomer. It had a melting point of 153-154\°.

\[ \sqrt{27} \alpha = +16.2^\circ \pm 0.4^\circ \]

Anal. Calcd. for \( \text{C}_{13} \text{H}_{15} \text{O}_{5} \text{N} \): neut. equiv., 133; N, 5.28.

Found: neut. equiv., 132, 134; N, 5.26, 5.28.

\#o-Carboxybenzoyl-\( \text{D} \)-valine

This compound was prepared from phthaloyl-\( \text{D} \)-valine by
the identical procedure used for the preparation of
o-carboxybenzoyl-\(\beta\)-valine. The melting point was 154-155\(^\circ\).

\[\left[\alpha\right]_D^{24} = -15.9^\circ \pm 0.5^\circ\]

Anal. Calcld. for C\(_{13}\)H\(_{15}\)O\(_5\)N: neut. equiv., 133; N, 5.28.

Found: neut. equiv., 132; N, 5.23

\textbf{cis-2-Carboxycyclobutanecarbonyl-\(\beta\L\)-valine}

The solid obtained from the hydrolysis of the corresponding imide was recrystallized several times from water to give a melting point of 178-179\(^\circ\).

Anal. Calcld. for C\(_{11}\)H\(_{17}\)O\(_5\)N: neut. equiv., 122; N, 5.76.

Found: neut. equiv., 123; N, 5.73, 5.73.

\textbf{cis-2-Carboxycyclobutanecarbonyl-\(\beta\D\)-valine}

The dried hydrolysis mixture was purified by solution in absolute ethanol, filtration from the inorganic matter, addition of ethyl acetate to prevent layering, and then addition of hexane to incipient precipitation. Several such purifications gave the amide in well-formed crystals; melting point 168-169\(^\circ\).

\[\left[\alpha\right]_D^{27} = +7.4^\circ \pm 0.3^\circ\]

Anal. Calcld. for C\(_{11}\)H\(_{17}\)O\(_5\)N: neut. equiv., 122; N, 5.76.

Found: neut. equiv., 123; N, 5.70, 5.71.
cis-2-Carboxycyclobutanecarbonyl-L-valine

This compound was synthesized and purified in the same manner used for the cis isomer. It also had a melting point of 168-169⁰.

\[ [\alpha]_D^{26} = -7.20 ^\circ + 0.40 \]

Anal. Calcd. for C\textsubscript{11}H\textsubscript{17}O\textsubscript{5}N: neut. equiv., 122; N, 5.76.

Found: neut. equiv., 122, 123; N, 5.73, 5.81.

Maleyl-DL-valine

The following procedure was adapted from that reported for the preparation of maleylglycine (94). A mixture of 10.9 g. (0.110 mole) of maleic anhydride and 11.7 g. (0.100 mole) of DL-valine was heated at 100⁰ for three-fourths of an hour during which time the powdery mass was often agitated. It was then cooled, triturated in very dilute hydrochloric acid, and filtered. This material was dissolved in 50 ml. of hot ethanol, 100 ml. of hot water was added, and the solution was allowed to crystallize in the cold. Filtration gave a yield of 15.5 g. (69%) of maleyl-DL-valine with a melting point of 165-167⁰. Two other recrystallizations from the same solvents produced a material with a melting point of 166-167⁰.


**Maleyl-D- and L-valine**

When the fusion of maleic anhydride and an optical isomer of valine was carried out at 100° as for the above racemic compound, an intractable oil resulted. A mixture of 5.8 g. (0.050 mole) of either D- or L-valine and 5.9 g. (0.060 mole) of maleic anhydride was kept at 70° and agitated until the mush changed to a solid. This required from fifteen to twenty minutes. The solid was dissolved in 200 ml. of hot ethyl acetate and then carbon tetrachloride was added to incipient precipitation. A seed was introduced and the mixture was set aside to crystallize in the cold. The yields were about 75% of theory. The melting points were 132-133° after several other recrystallizations from the same solvents.

**Maleyl-D-valine**

\[ \alpha \frac{25}{D} = +1.6° \pm 0.1° * \]

\[ \alpha \frac{21}{D} = -26.2° \pm 0.3° ** \]


* Rotation was taken on a 3% solution in absolute ethanol.

** Rotation was taken on a 3% solution in ethyl acetate.
Found: neut. equiv., 108; N, 6.45.

**Maleyl-L-valine**

\[ \alpha^{21}_D = +25.5^\circ \pm 0.5^\circ \]


Found: neut. equiv., 109; N, 6.50.

**Fumaryl-di-DL-valine**

Eleven and seven-tenths g. (0.10 mole) of DL-valine was dissolved in 100 ml. (0.10 mole) of 1.0 N sodium hydroxide. To this solution there were added, with vigorous shaking and in several alternate portions, 7.7 g. (0.050 mole) of fumaryl chloride** dissolved in 50 ml. of ether and 100 ml. (0.10) of 1.0 N sodium hydroxide. Each solution had been cooled in ice. After the odor of fumaryl chloride had disappeared, the aqueous layer was separated and acidified with concentrated hydrochloric acid. The resulting precipitate was filtered off and dried. It was recrystallized by solution in 200 ml. of boiling methanol followed by the addition of 200 ml. of warm water. After cooling overnight in the refrigerator there was obtained 10 g. (64% of theory) of fumaryl-di-DL-valine with a melting point*** of 266-267°. The melting point*** rose to 282-283°

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* Rotation was taken on a 3% solution in ethyl acetate.
** A white label product of the Eastman Kodak Company, Rochester, New York, was used.
*** A copper block was used.
after several other recrystallizations from the same solvents.

Anal. Calcd. for $C_{14}H_{22}O_{6}N_2$: neut. equiv., 157; $N$, 8.91.

Found: neut. equiv., 158; $N$, 8.70.

Itaconic anhydride

The destructive distillation of citric acid and the hydrolysis of the crude intermediate itaconic anhydride to the acid have been described (95), but a method for the isolation and purification of the anhydride was omitted. The main contaminant is the isomeric citraconic anhydride.

The following procedure proved to be convenient for the purification of the itaconic anhydride: The anhydride layer, resulting from the distillation of citric acid, was dissolved in chloroform and dried over sodium sulfate. The chloroform was evaporated off in vacuo, and a small quantity of ether was slurried with the resulting oil. Crystals of itaconic anhydride formed, and the liquid citraconic anhydride dissolved in the ether. The itaconic anhydride could be further purified from ether-hexane; melting point 65-66°.

Citraconic anhydride

The ethereal mother liquor from the itaconic anhydride

was evaporated and the residue was distilled, first at atmospheric pressure and then in vacuo (96) to yield a quantity of citraconic anhydride.

**Ethyl itaconyl-DL-valinate**

A solution of 23.5 g. (0.210 mole) of itaconic anhydride in anhydrous ether was added to a dried ether solution of the free base prepared from 44.0 g. (0.242 mole) of DL-valine ethyl ester hydrochloride. The total volume was about 500 ml. After the solution had stood for three days, the ether was evaporated off, and the residue was triturated in cold water until it solidified. Filtration gave 37.5 g. (70% of theory) of a product with a melting point of 79-83°. Repeated recrystallization from hot water finally gave the ester with a melting point of 88-89°. It was not determined whether the methylene group is in the α or β position relative to the amide group.

Anal. Calcd. for C₁₂H₁₉O₅N: neut. equiv., 257; N, 5.44.

Found: neut. equiv., 256; N, 5.44.

**Itaconyl-DL-valine**

One gram (0.0039 mole) of the above ethyl itaconyl-DL-valinate was dissolved in 8.0 ml. (0.01 mole) of 1.85 N

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sodium hydroxide and the solution was allowed to stand for five hours. It was then acidified and evaporated to dryness **in vacuo** at room temperature. The residue was extracted several times with hot absolute ethanol. Evaporation of the alcohol left a residue which soon crystallized; melting point 123-128°. This material was best purified from water in which it was very soluble. Following several recrystallizations the melting point rose to 139-140°.

**Anal.** Calcd. for $\text{C}_{10}\text{H}_{15}\text{O}_{5}\text{N}$: neut. equiv., 115; N, 6.11.

Found: neut. equiv., 115; N, 6.04.

**endo-cis-3,6-Endomethylene-$\Delta^4$-tetrahydrophthaloyl-DL-valine**

Eight and two-tenths g. (0.050 mole) of **endo-cis-3,6-endomethylene-$\Delta^4$-tetrahydrophthalic anhydride** and 5.8 g. (0.050 mole) of $\text{DL}$-valine were fused at 150° until all water was removed. This required about twenty minutes. Several recrystallizations from carbon tetrachloride-hexane gave crystals with a melting point of 118-119°.

**Anal.** Calcd. for $\text{C}_{14}\text{H}_{17}\text{O}_{4}\text{N}$: neut. equiv., 263; N, 5.32.

Found: neut. equiv., 264, 265; N, 5.39.

**endo-cis-3,6-Endomethylene-$\Delta^4$-tetrahydrophthaloyl-DL-valine**

* This compound is sold by the Eastman Kodak Company under the name "bicyclo (2,2,1) 5-heptene-2,3-dicarboxylic anhydride."
Eight and two-tenths g. (0.050 mole) of endo-cis-3,6-endomethylene-Δ4-tetrahydrophthalic anhydride was fused with 5.8 g. (0.050 mole) of L-valine as described above for the racemic isomer. After repeated recrystallization from benzene-cyclohexane there were obtained crystals with a melting point of 116-117°. In view of the instability of the anilide in aqueous solution (see below), no attempt was made to hydrolyze to the di-acid either this or the preceding imide. At the almost neutral pH used for bacteriological testing, the corresponding phthalamic acids would very likely cyclize to the imides.

\[ \delta_24^D = +60.4^\circ \pm 0.5^\circ \]


Found: neut. equiv., 264; N, 5.35.

**N-Phenyl-endo-cis-3,6-endomethylene-Δ4-tetrahydrophthalimide**

This compound has been previously synthesized by a longer procedure (97). It was more readily prepared by the following method: Eight and two-tenths g. (0.050 mole) of endo-cis-3,6-endomethylene-Δ4-tetrahydrophthalic anhydride and 5.1 g. (0.055 mole) of aniline were heated together for twenty minutes at 150°, by which time all water had been

removed. The residue was dissolved in 50 ml. of ethanol, and then 70 ml. of hot water was added. After cooling, the crystals were filtered off to give a yield of 11.5 g. (96% of theory) with a melting point of 141-143°. A second recrystallization from the same solvents raised the melting point to 142-144°. The amount of product recovered was 10.2 g. (86% of theory). The above reference reported a melting point of 144°.

**endo-cis-3,6-endo-methylene-Δ⁴-tetrahydrophthalanilic acid**

Earlier experiments to prepare this compound were reported to be unsuccessful (97) and produced only the imide. The failure was probably due to the use of hot acetic acid for purification of the reaction product between aniline and the bicyclic anhydride.

To a solution of 4.1 g. (0.025 mole) of *endo-cis*-3,6-endo-methylene-Δ⁴-tetrahydrophthalic anhydride in 20 ml. of benzene there was added, at room temperature, a solution of 2.5 ml. (0.030 mole) of aniline in 10 ml. of benzene. Crystals of the anilide soon appeared and after being cooled, they were filtered off to give a good yield of the desired acid. These crystals were purified at room temperature from ethyl acetate-hexane to a melting point of 135-145°, depending upon the rate of heating.

The anilide rapidly formed the imide in aqueous solution. This is indicated by the fact that following neutralization by sodium hydroxide to a phenolphthalein end-point
the solution soon became more basic, even when kept in ice.

Anal. Calcd. for C\textsubscript{15}H\textsubscript{15}O\textsubscript{3}N: neut. equiv., 257; N, 5.44.

Found: neut. equiv., 261; N, 5.43.

4-Hydroxy-cis-2,6-endomethylenehexahydrophthalic acid γ-lactone

Alder and Stein (98) reported the preparation of this compound by shaking the unsaturated bicyclic anhydride with 50\% sulfuric acid at room temperature. The following modification proved to be more convenient: Forty g. (0.241 mole) of endo-cis-3,6-endomethylene-Δ⁴-tetrahydrophthalic anhydride was added to 400 ml. of 50\% sulfuric acid by volume, which was at 50-60°. The mixture, which soon became a solution, was stirred at room temperature for four hours. It was then poured into 400 ml. of ice-water, cooled, and filtered. The moist material was recrystallized from 200 ml. of water, giving a yield of 35 g. (79\% of theory) of the lactonic acid with a melting point of 195-200°. It was further recrystallized to the reported melting point of 203°.

2-Carboxy-4-hydroxy-cis-3,6-endomethylenehexahydrobenzanilide γ-lactone

These two experiments were carried out to determine the reactivity of the lactone ring toward amines.

A mixture of 0.50 g. (0.0027 mole) of the above lactonic acid and 0.70 g. (0.0034 mole) of phosphorus pentachloride was agitated in 10 ml. of benzene until all the solids were in solution. Dry air was passed through this solution to remove the hydrogen chloride gas, and then a cold solution of 2.0 ml. (0.021 mole) of aniline in 10 ml. of benzene was added. After the mixture had stood at room temperature for fifteen minutes, the solid material was filtered off and slurried successively with dilute hydrochloric acid, dilute sodium carbonate solution, and finally with water. The anilide, having a melting point of 232-233°, was obtained after several recrystallizations of the residue from butanol.

Anal. Calcd. for C15H15O3N:  N, 5.44.
Found:   N, 5.46.

The anilide was also obtained by the fusion of the lactonic acid with aniline at 200° for two hours. This indicates that, due to the fixed ring system, the lactone ring is extremely unreactive toward amines.

Methyl ester of 2-carboxy-4-hydroxy-cis-2,6-endomethylene-hexahydrobenzoyl-DL-valine γ-lactone

The acid chloride of the previously described cis-lactonic acid was prepared in 15 ml. of benzene from 3.6 g.
(0.020 mole) of the acid and 4.6 g. (0.022 mole) of phosphorus pentachloride. The benzene and phosphorus oxychloride were removed from the solution in vacuo while it was in a water-bath kept at 60°. To the cooled residue there was added a cold benzene solution of the base prepared from 8.3 g. (0.050 mole) of DL-valine methyl ester hydrochloride. The mixture was allowed to stand at room temperature for several hours, and it was then extracted once with water, with dilute hydrochloric acid, and finally with dilute sodium carbonate solution. The benzene solution was dried over sodium sulfate and then evaporated in vacuo, leaving a residue of 4.3 g. (73% of theory) of the desired methyl ester. Repeated purification from chloroform-hexane gave a product with a melting point of 168-169°.

Found: N, 4.71.

2-Garboxy-4-hydroxy-oia-3,6-endomethylenehexahydrobenzoyl-
DL-valine γ-lactone

Two and eight-tenths g. (0.0095 mole) of the above methyl ester was added to 9.8 ml. (0.020 mole) of 1.95 N sodium hydroxide, and the mixture was warmed on the steam-bath for a few minutes until a complete solution was obtained. This solution was cooled, and then acidified with concentrated hydrochloric acid. After being kept cold for awhile, the mixture was filtered to give 2.4 g. (89% of
theory) of material with a melting point of 187-189°. The melting point rose to 191.5-192.5° after three recrystallizations from water.

Anal. Calcd. for C_{14}H_{19}O_{5}N: neut. equiv., 281; N, 4.98.

Found: neut. equiv., 280; N, 4.94.

Methyl ester of 2-carboxy-4-hydroxy-cis-3,6-endomethylene-hexahydrobenzoic acid 7-lactone

Alder and Stein (99) isolated this ester in a 15% yield following esterification with sulfuric acid as a catalyst. The use of hydrogen chloride proved to be more satisfactory.

A mixture of 20 g. (0.11 mole) of the previously described cis-lactonic acid and 200 ml. of methanol was saturated with dry hydrogen chloride and refluxed for one hour. It was then resaturated with hydrogen chloride and refluxed for a total of three hours. The residue, resulting from evaporation of the methanol in vacuo, was dissolved in chloroform and extracted once with water and once with potassium carbonate solution. The chloroform solution of the ester was dried over sodium sulfate and evaporated to

(99) Alder and Stein, Ann., 514, 24 (1934).
give a residue of 18.5 g. (86% of theory) of the methyl ester with a melting point of 80-82°C. The melting point was raised to 82-83°C after several recrystallizations from ethyl acetate-hexane. That previously reported (99) was 85°C.

2-Carboxy-4-hydroxy-trans-3,6-endomethylenehexahydrobenzoic acid γ-lactone

This acid was prepared from the 2-carboxy-4-hydroxy-cis-3,6-endomethylenehexahydrobenzoic acid γ-lactone according to the procedure of Alder and Stein (99). The inversion of the carboxyl group was effected by refluxing the methyl ester of the cis-acid with a solution of sodium methy late in methanol. Alkaline hydrolysis then gave the trans-acid in an excellent yield.

2-Carboxy-4-hydroxy-trans-3,6-endomethylenehexahydrobenzoyl-DL-valine γ-lactone

The acid chloride of the above trans-lactonic acid was prepared in benzene from 5.0 g. (0.027 mole) of the acid and 6.0 g. (0.029 mole) of phosphorus pentachloride. The benzene and the phosphorus oxychloride were removed from the solution in vacuo while it was in a water bath which was kept at 60°C. Small amounts of benzene were then added and evaporated to insure that all of the phosphorus oxychloride was removed. To the residue there was added, with vigorous shaking, a cold solution of 6.5 g. (0.055
mole) of DL-valine in 50 ml. (0.10 mole) of 2 N potassium carbonate. The resulting solution was acidified in the cold with concentrated hydrochloric acid. Upon the addition of about 20 ml. of ethyl acetate to the oily mixture, there appeared a precipitate which was filtered off and dried; melting point 210-218°. The material was purified at room temperature by solution in absolute ethanol, addition of ethyl acetate to prevent layering, and then precipitation with hexane. Repetition of this procedure finally gave crystals with a melting point of 230-231°.

Anal. Calcd. for C_{14}H_{19}O_{5}N: neut. equiv., 281; N, 4.98.

Found: neut. equiv., 281; N, 4.88.

Miscellaneous Preparations

**DL-Valine methyl ester hydrochloride**

A mixture of 30 g. of DL-valine and 300 ml. of methanol was saturated with dry hydrogen chloride. It was refluxed for two hours, resaturated with hydrogen chloride, and refluxed for fifteen minutes longer. Evaporation in vacuo on a steam bath gave the methyl ester hydrochloride as an oil which crystallized only after the complete removal of the excess hydrogen chloride. The resulting solid was considered pure enough for further reactions.

For analytical purposes the material was purified. The
following method of purification was the only one discovered which did not cause oiling: solution in acetone, addition of chloroform, and precipitation by hexane. The melting point remained in the range 90-97°.

Anal. Calcd. for C₆H₁₄O₂NCl: N, 8.36; Cl, 21.2.

Found: N, 8.44; Cl (gravimetric), 21.4, 21.4.

**DL-Valine n-butyl ester sulfate**

The n-butyl ester of DL-valine was prepared by a Fischer esterification according to the procedure of Morgan (100). This ester was carefully added to a solution of concentrated sulfuric acid in anhydrous ether until the oil, which first formed, had solidified. The solid was recrystallized from acetone-ether to a melting point of 140.5-141.5°.


Found: N, 6.15; SO₄ (gravimetric), 21.6, 21.7.

**Acetyl-DL-valine**

This procedure is an adaption of a method given for the preparation of aceturic acid (101). A slurry of 11.7 g. (0.10 mole) of DL-valine and 50 ml. of water was warmed to 50° and then 30 ml. (0.32 mole) of acetic anhydride was

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added in one portion. The mixture was vigorously shaken until all the valine was in solution, and then it was allowed to stand for thirty minutes. The solution was evaporated to dryness in vacuo, and the residue was recrystallized once from 80 ml. of water to give 9.5 g. (60% of theory) of crystals with a melting point of 143-145°. Repeated crystallization from acetone raised the melting point to 147.5-149°. A melting point of 144-146° has been reported for a product obtained by the action of acetic anhydride on DL-valine in alkaline solution (102).

Anal. Calcd. for C7H13O3N: neut. equiv., 159(177,449),(1055,705); N, 8.80.

Found: neut. equiv., 158, 159; N, 8.77.

**Benzoyl-DL-valine methyl ester**

Twenty grams (0.091 mole) of benzoyl-DL-valine (103) was added to 200 ml. of methanol and the mixture was saturated with dry hydrogen chloride. This solution was refluxed for one-half hour and the methanol was then completely removed by evaporation in vacuo. After the addition of 200 ml. of cyclohexane and warming to effect complete solution, a crystal of the desired methyl ester was introduced, and the mixture was set aside to cool and crystallize. A yield of

20 g. (94% of theory) of the desired methyl ester of benzoyl-
DL-valine was obtained; melting point 86-89°. The melting
point rose to 90-91° after several recrystallizations from
ethanol-water.

Anal. Calcd. for C_{13}H_{17}O_3N: N, 5.95.

Found: N, 6.02.

N-(n-butyl)-phthalamic acid

Fourteen and seven-tenths g. (0.10 mole) of phthalimide
and 7.3 g. (0.10 mole) of n-butylamine were fused at 130°
for one-half hour. The liquid N-butylphthalimide (104) was
not purified, but was hydrolyzed directly to the desired
acid as follows: To the imide there were added 10 ml. of
ethanol and 50 ml. (0.10 mole) of 2 N sodium hydroxide.
The mixture was heated to boiling for several minutes until
a test sample produced no turbidity upon dilution with water.
It was then cooled and precipitated with concentrated hydro-
chloric acid. The yield was 17 g. (77% of theory) of a
product with a melting point of 105-106°. Because recrystal-
lization from hot ethanol-water gave an impure product, the
acid was purified at room temperature by solution in ether
and precipitation as crystals by hexane to a melting point
of 107.5-108.5°.

4, No. 8, 405 (1939). Original not seen. Abstracted
Anal. Calcd. for $C_{12}H_{15}O_3N$: neut. equiv., 221; N, 6.33.

Found: neut. equiv., 222, 223; N, 6.29, 6.32.

cis-1,2-Cyclobutanedicarboximide

This compound has been previously described by Menon and Simonsen (105), but their directions were not complete. cis-1,2-Cyclobutanedicarboxylic anhydride was melted in an oil bath at 135° and was saturated with dry ammonia gas. The oil-bath temperature was then raised to 180° and held there for one-half hour. The resulting imide was nicely recrystallized from butanol-hexane to a melting point of 134.5-135.5°. Menon and Simonsen reported a melting point of 121°. No pure material could be obtained using their method of purification from chloroform-hexane.


Found: N, 11.3.

cis-1,2-Cyclobutanedicarboxylic acid

To 3.0 g. (0.24 mole) of cis-1,2-cyclobutanedicarboxylic anhydride cooled in an ice-bath, there was added 6 ml. of cold concentrated ammonium hydroxide. After all the anhydride had gone into solution, the excess ammonia was volatilized in vacuo with the aid of a capillary tube. Excess

concentrated hydrochloric acid was added, and after cooling for awhile, the precipitated amide and ammonium chloride were filtered off. The weight was 3.0 g. (88% of theory). Several recrystallizations from ethanol gave the acid with a melting point of 159.0-159.5°.


Found: neut. equiv., 144, 145; N, 9.76, 9.79.

Resolution of DL-valine by the brucine salt method.

The formylation procedure was that of du Vignaud, Dorfmann, and Loring (106), as adapted to valine by Fling (20). The resolution was that of Emil Fischer (107) but with one convenient and time-saving modification, the formyl-D- or L-valine was not isolated but was hydrolyzed to the free amino acid after decomposition of the brucine salt.

One hundred g. (0.85 mole) of DL-valine and 1500 ml. (24 mole) of 88-90% formic acid were placed in a 3-l. flask equipped with a stirrer, dropping funnel, and thermometer. The solution was heated to 60°, the source of heat was removed, and with stirring, 500 ml. (5.3 mole) of acetic

(107) Fischer, Ber., 39, 2320 (1906).
anhydride was dropped in so that the temperature remained in the range 58-62°. Stirring was continued for fifteen minutes after the addition of the acetic anhydride was complete, and then 250 ml. of water was added. The mixture was evaporated in vacuo on a steam bath until crystals appeared and it was then placed in a refrigerator overnight. Filtration gave 77 g. (62% of theory) of formyl-DL-valine with a melting point of 137-143°. (Fischer reported a melting point of 139-144° for a purified sample). The filtrate was evaporated to dryness and the residue was triturated in 50 ml. of cold 5% hydrochloric acid. The remaining solid was formyl-DL-valine, having a weight of 23 g. and a melting point of 133-138°. The total yield of formyl-DL-valine was therefore 100 g. (81% of theory).

One hundred g. (0.69 mole) of formyl-DL-valine and 275 g. (0.70 mole) of brucine were dissolved in 3000 ml. of boiling methanol, about 5 g. of charcoal was added, and after stirring briefly the mixture was filtered. It was allowed to stand in ice for four hours, and then the precipitate of brucinium formyl-DL-valinate was filtered off. (The hot methanol solution was seeded to insure crystallization of the salt. The seed was obtained by cooling a small portion of the solution.) The precipitate was dried and weighed to check that the theoretical amount, 187 g., was obtained. The filtrate was evaporated in vacuo to
dryness in a water-bath kept at 60°. The residue was brucinium-formyl-L-valinate.

Each isomeric brucinium salt was decomposed in the same manner. The salt was dissolved in one l. of ice-water, and 2 N lithium hydroxide was carefully added until the solution was basic. The crystallization of the brucine was facilitated by stirring. The mixture was allowed to stand in ice for ten minutes and then filtered through a large Buchner funnel as rapidly as possible. The crystals of brucine were well washed with ice-water. The filtrate was extracted once with chloroform and twice with ether to remove traces of brucine. It was then acidified with 60 ml. of concentrated hydrochloric acid and heated in vacuo with a capillary to remove all the ether fumes. This was as a safeguard for the next operation.

The solution was then distilled at atmospheric pressure with a Meker burner until the volume was 150-200 ml. The time required was about three hours. The solution was cooled and was adjusted to pH 6.0 with 2N lithium hydroxide using a glass electrode. This solution was again evaporated to a volume of 150-200 ml. but this time in vacuo on the steam-bath. Fifteen hundred ml. of absolute ethanol was added, and the mixture was cooled overnight. Filtration gave about 25 g. (64% of theory based on formyl-L-valine) of each isomer, which was 93-98% optically pure as indicated by determination of the specific rotation. The isomers
were recrystallized by solution in the least amount of hot water and addition of absolute ethanol. Rotations observed on 3% solutions of purified D- and L-valine in 6 N hydrochloric acid are the following (107):

\[
\begin{align*}
L\text{-valine} & \quad [\alpha]_D^{20} = +28.8^\circ \\
D\text{-valine} & \quad [\alpha]_D^{20} = -29.0^\circ
\end{align*}
\]

**Purification of recovered brucine**

Recovered brucine was dissolved in boiling 5% sulfuric acid, charcoal was added, and after being stirred well the mixture was filtered. Brucine was precipitated from the still warm solution by the cautious addition of ammonium hydroxide. It was filtered off after the mixture had been cooled in ice. This brucine was in the form of a hydrate and had to be dried at an elevated temperature to insure complete dryness. This was accomplished by air-drying and then heating in an oven at 115° for several hours. The melting point of brucine·4H₂O is 105° and that of anhydrous brucine is 178°.

**Attempted enzymic resolution of DL-valine**

Attempts were made to resolve DL-valine by the papain-catalyzed precipitation of acylated L-valinanilides. This method was developed by Bergmann and Fraenkel-Conrat (108) for other amino acids.

All of the acylated valines tested proved to be extremely unreactive in spite of the structural similarity of valine to leucine, which gave a quantitative precipitation of benzoyl-L-leucinanilide under the conditions used.

The general procedure for the papain-catalyzed precipitations was as follows: In 50 ml. test tubes there were placed 0.025 g. of commercial papain*, 0.025 g. of cysteine hydrochloride, 7.5 ml. of 1 M citric acid solution and 0.0025 mole of the desired acylated valine. Five tenths ml. (0.0055 mole) of aniline was added, and the mixture was adjusted to a pH of 4.5 with concentrated sodium hydroxide and to a final volume of 25 ml. with water. Each tube was then stoppered and heated in a water bath at 40° for four days, at which time the precipitate, if any, was filtered off. The precipitates of the anilides were washed with dilute potassium carbonate solution and then with water. They were dried and weighed. Controls of either benzoyl-DL-leucine or hippuric acid were included to check on the activity of the enzyme. The former always gave a quantitative yield of benzoyl-L-leucinanilide and the latter gave a 7-14% yield of benzoylglycinanilide. All of the acylated valines except phthaloyl-DL-valine dissolved completely in the reaction mixture.

* A product of the Nutritional Biochemicals Corporation, Cleveland, Ohio.
Benzoyl-DL-valine gave a 4% yield of benzoyl-L-valinilide; forvaline, acetyl-DL-valine, and phthaloyl-DL-valine produced no detectable amount of anilide. Although the pH of the reaction medium does have a large effect in determining the yields of anilides (109), it was not considered likely that varying the pH would induce a quantitative precipitation of benzoyl-L-valinilide. On the basis of these experiments it appeared that DL-valine could not be resolved by this method. The optical isomers of valine used in this thesis were obtained by the previously described brucine salt method.

**N-Phenylphthalimide precipitations**

When o-carboxybenzoyl-DL-valine was used as a substrate in the above manner, there was obtained a substance which was identified as N-phenylphthalimide by analysis and mixed melting point with a known sample. The yield was 42-43% of theory, based on the assumption that both the D and L isomers react equally well. A control without papain gave a 50% yield of N-phenylphthalimide, indicating that the enzyme exerted no catalytic effect.

To continue the survey of this unexpected reaction,

* Purified and analyzed by Jaquetta S. Halverson. It had a melting point of 211-212°C. Calcd. for C_{18}H_{20}O_{2}N: N, 9.46. Found: N, 9.37, 9.44.

other phthalamic acids were tested using these amounts of materials: the phthalamic acid (0.005 mole), 0.93 ml. (0.01 mole) of aniline, 22.5 ml. of 1 M sodium citrate solution (pH 4.5) and 22.5 ml. of water. The weights of \( N \)-phenylphthalimide which were filtered off from duplicate tubes after heating at 40° for seven days were as follows, the percentages of theory being in parentheses:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Weight (g)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phthalamic acid</td>
<td>0.76</td>
<td>(69%)</td>
</tr>
<tr>
<td>( N )-butylphthalamic acid</td>
<td>0.82</td>
<td>(74%)</td>
</tr>
<tr>
<td>( N )-phenylphthalamic acid</td>
<td>0.25</td>
<td>(23%)</td>
</tr>
<tr>
<td>( \alpha )-carboxybenzoyl-( DL )-valine</td>
<td>0.91</td>
<td>(82%)</td>
</tr>
</tbody>
</table>

The \( N \)-phenylphthalamic acid was the only compound which did not go completely into solution after combining and shaking the reaction mixture.

No conclusions as to the mechanism for the formation of \( N \)-phenylphthalimide can be drawn from these limited data, although it is not improbable that \( N \)-phenylphthalamic acid is a reaction intermediate. The low yield from \( N \)-phenylphthalamic acid itself may be due to its insolubility in the aqueous medium. This reaction was not further investigated because of its remoteness from the problem. It might possibly be of interest as a model for \textit{in vitro} peptide bond synthesis.
DISCUSSION

It may be considered (110) that a bactericidal substance interferes with an enzymic process for which there is no alternate pathway, and that a bacteriostatic substance interferes with the main pathway but leaves open minor ones which can support life at a reduced rate of growth. There are four general ways by which a substance may interfere with the action of an enzyme*: reaction with the enzyme protein, reduction of the activity of the coenzyme or activator, competition with the substrate for an active center on the enzyme, and finally, reduction in the activity of the substrate. As examples of these methods there may be mentioned the reaction of bis-β-chloroethylsulfide with sulfhydryl groups in the protein portion of urease (111), the action of hydrogen cyanide on iron-porphyrin coenzymes (112), the competition between succinic acid and malonic acid for succinic acid dehydrogenase (113), and the reaction of formaldehyde with a free amino group in the peptide substrate.

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(113) Quastel and Wooldridge, Biochem. J., 22, 689 (1928).
With the above methods as a basis, many simple acyl groups would assume a new importance antibacterially if they could be brought near to an enzyme system which is essential for bacterial life. It is conceivable that coupling these groups with a naturally occurring amino acid would enable them, after penetration of the bacterial cell, to gain proximity to a particular enzyme system and to exert an inhibiting effect. Because previous work (20,22) indicates that the mere inclusion or repetition of a D configuration in an amino acid derivative is not enough to confer antibiotic activity, emphasis was placed upon the finding of active acyl groups prior to the study of the effect of optical isomerism. It was with these thoughts in mind that certain compounds containing amino acids were selected and prepared.

An amino acid derivative having unusual bond distances or angles might possibly interfere with enzyme-substrate complex formation and thus interfere with bacterial metabolism. For this reason the cis-2-carboxycyclobutanes-carbonylvalines were prepared. Kilpatrick and Spitzer (115) have calculated that the angle between two hydrogen atoms

(114) Anson, Science, 81, 467 (1935).
attached to the same carbon atom in the ring of cyclo-
butane is approximately six degrees greater than the usual
tetrahedral angle of $109^\circ 28'$. The distance between the
ring carboxyl and the amide group may therefore be con-
sidered as a sort of "unnatural" distance. Structures of
this type, involving slight variations in bond angles and
distances, do not appear to have been previously incor-
porated into amino acid derivatives for this purpose.

Various acylated valines were prepared which contain
an ethylenic linkage $\alpha,\beta$ to a carbonyl group. There are
clear indications that such activated ethylenic linkages
can inactivate certain enzymes by adding their sulfhydryl
groups. By thus reacting with essential enzymes, bacterial
metabolism might be inhibited. Geiger and Conn (116,117)
have shown that an increase in the size of groups adjacent
to the ethylenic bond in various acrylophenones may be
correlated with a decrease in antibacterial activity. On
this basis, of the compounds maleyl-, itaconyl-, and
fumaryl bis-valine, the itaconyl valine with its terminal
methylenegroup is the most interesting.

Alder and Stein (118) have established that the adduct
of cyclopentadiene with maleic anhydride,

(118) Alder and Stein, Ann., 504, 216 (1933).
endo-cis-3,6-endomethylene- $\Delta^4$-tetrahydrophthalic anhydride, has the structure shown, and by a series of reactions they have converted it into the trans-lactonic acid. Because of the gross similarity to the ring structure of penicillin, which was determined by X-ray crystallography (119), this

trans-lactonic acid was coupled with valine. The isomeric compound containing the cis-lactonic acid was also prepared.

The imide formed by the fusion of endo-cis-3,6-endomethylene- $\Delta^4$-tetrahydrophthalic anhydride with amylamine has been patented (120) as a pyrethreum substitute in insect spray. Because the ring system does show such decided biological activity, the imide formed between the anhydride and valine was prepared to be tested against bacteria.

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(120) United States Patent 2, 424, 220.
SUMMARY

A number of acyl derivatives of valine were prepared for bacteriological testing. They are the following: phthaloyl-\(DL\)-valine and monohydrate, \(O\)-carboxybenzoyl-\(DL\)-, \(D\)-, and \(L\)-valine, \(cis\)-1,2-cyclobutanedicarbonyl-\(DL\)-valine and monohydrate, \(cis\)-1,2-cyclobutanedicarbonyl-\(D\)- and \(L\)-valine, \(cis\)-2-carboxycyclobutanecarbonyl-\(DL\)-, \(D\)-, and \(L\)-valine, maleyl-\(DL\)-, \(D\)-, and \(L\)-valine, fumaryl-\(DL\)-valine, itaconyl-\(DL\)-valine, \(endo\)-\(cis\)-3,6-endomethylene-\(\Delta^4\)-tetrahydrophthaloyl-\(DL\)- and \(D\)-valine, 2-carboxy-4-hydroxy-\(cis\)-3,6-endomethylenhexahydrobenzoyl-\(DL\)-valine-lactone, 2-carboxy-4-hydroxy-\(trans\)-3,6-endomethylenhexahydrobenzoyl-\(DL\)-valine \(\gamma\)-lactone.

The following new compounds were also prepared: methyl itaconyl-\(DL\)-valinate, methyl 2-hydroxy-4-carboxy-\(cis\)-endomethylenhexahydrobenzoyl-\(DL\)-valinate \(\gamma\)-lactone, \(DL\)-valine methyl ester hydrochloride, benzoyl \(DL\)-valine methyl ester, \(DL\)-valine \(n\)-butyl ester sulfate, \(N\)-\(n\)-butylphthalamic acid, \(cis\)-1,2-cyclobutanedicarboxylic acid, \(endo\)-\(cis\)-3,6-endomethylene-\(\Delta^4\)-tetrahydrophthalanic acid, and 2-carboxy-4-hydroxy-\(cis\)-3,6-endomethylenhexahydrobenzanilide \(\gamma\)-lactone.

Attempts to resolve \(DL\)-valine by the papain-catalyzed precipitation of acylated \(L\)-valinamides were unsuccessful from a practical viewpoint.
An interesting conversion of phthalamic acids into 
N-phenylphthalimide is reported.
ACKNOWLEDGMENTS

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