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Approach to the Use of Benzylpenicillinacylase for Configurational Correlations of Amino Compounds

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Benzylpenicillinacylase (BPA) from *Escherichia coli* ATCC 9637 was found capable of hydrolyzing, in addition to the *N*-acyl derivatives of α-amino acids, the *N*-phenylacetyl derivatives of a variety of primary amino compounds. An approach to the use of the stereospecific action of BPA for correlating the absolute configurations of amino compounds is described. For this purpose enzymatic hydrolysis of several *N*-phenylacetylamino derivatives with known absolute configuration was examined. Reference to a single stereomodel was made to analyze hydrolytic data. The substituents at the asymmetric carbon atoms of the preferred enantiomers were then classified in terms of position occupied inside groups of priority sequences. The priority relations found constitute an empirical guide for stereochemical predictions. For some of the substrates examined the absolute configuration was determined, in the course of the present work, by chemical methods.

The stereospecific action of enzymes of the class of the acylases, amidases, decarboxylases, and oxidases is a long-established and reliable method of determining the absolute configuration of α-amino acids. More recently, benzylpenicillinacylase (BPA) from *Escherichia coli* ATCC 9637 has been reported to show L-directed stereochemical preference in hydrolyzing *N*-phenylacetylamino acids.[2-4] In accordance with this observation we have used BPA to define and confirm the absolute configuration of some amino acids.[3,4]

On further investigation[5] BPA was found capable of (i) hydrolyzing (in addition to the *N*-phenylacetylamino acids) a variety of *N*-phenylacetylamino compounds with a primary amino group and (ii) reacting at different rates on both the enantiomers of racemic mixtures.

On the basis of these properties it seems interesting to examine the potentiality of this enzyme in the field of enzymatic hydrolysis, particularly for the resolution of racemates and for configurational correlations.

Although in the case of the enzymatic hydrolysis of *N*-acylamino acids, the configurational correlations can be defined by referring the results to the D/L system, it is evident that this assumption cannot be retained in the more general case of the enzymatic hydrolysis of *N*-acylamino compounds. As a continuation of previous work,[5] this paper presents an attempt to correlate the absolute configuration of amino compounds, by using the BPA-catalyzed hydrolysis of the amide linkage. For this purpose several *N*-phenylacetylamino compounds having known absolute configuration were tested with the acylase. Hydrolysis results of all examined substrates were then analyzed by using a single model, corresponding to the more rapidly hydrolyzed enantiomer. The relative positions of the substituents at the asymmetric carbon atom of the preferred enantiomer were examined, in order to define a method for correlating the absolute configurations.

Results and Discussion

All substrates subjected to enzymatic hydrolysis with BPA are reported in Table I (the more rapidly hydrolyzed enantiomers are also shown). Experimental details relative to the hydrolysis of compounds **1–25** were reported in previous communications.[3-6] Hydrolysis conditions and results relative to substrates examined here are summarized in Table II. *N*-Phenylacetyl derivatives were generally prepared following known methods. In the case of *N*-phenylacetylserinonitrile (**29**) and *N*-phenylacetyl-4-cyano-4-phenylacetamidobutyric acid (**32**), the syntheses were accomplished starting from (2-tetrahydropyranyloxy)acetaldehyde and 2-ketoglutaric acid, respectively. Hydrolysis experiments were performed with a purified preparation of BPA. The reactions were carried out limiting the time to avoid the complete hydrolysis of racemic substrates. The unaltered portion of the *N*-phenylacetyl derivatives was isolated from the reaction mixture and the optical activity examined (Table II). The progress of the hydrolysis was followed by determining the phenylacetic acid produced by GC.

In order to establish the stereochemical preference of the enzyme, we have determined the absolute configuration of the substrates through chemical correlation. In the case of the *N*-phenylacetyl derivatives **28, 30, 34,** and **35,** whose corresponding amino compounds have known absolute configuration, the configurational correlations were simply established by preparing the *N*-phenylacetyl derivatives of the optical active amino compounds. In the case of substrates **26, 27, 29,** and **32,** the corresponding amino compounds have unknown absolute configuration. The optically active *N*-phenylacetyl derivatives recovered from the enzymatic hydrolyses were then transformed by chemical methods into compounds having known absolute configuration, as reported in Table III. In the case of the methyl esters **31** and **33,** the absolute configurations of the corresponding acids **8** and **32** are known (the configuration of **32** was defined by us, as reported in Table III). Corresponding optically active acids were then esterified to establish the desired correlation.

To define a method for stereochemical correlations, the following approach was adopted. A single model,[7] reported in Figure 1, was defined and used to represent the configuration at the chiral center of the more rapidly hydrolyzed enantiomers. Since the absolute configuration of the enantiomer

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Table I. Compounds Subjected to Enzymatic Hydrolysis and Absolute Configuration of the Preferred Enantiomers

Compd[h]	Registry no.	Preferred enantiomer: Absolute[a] config	Registry no.	Positions of substituents in model of Figure 1: A	B
N-PA-alanine (1)	17966-65-3	(*S*)-(−)[b]	718-07-0	COOH	CH_3
N-PA-2-aminobutyric acid (2)	65451-16-3	(*S*)-(−)[c]	836-32-8	COOH	C_2H_5
N-PA-valine (3)	2752-50-3	(*S*)-(−)[b]	725-67-7	COOH	$CH(CH_3)_2$
N-PA-leucine (4)	65415-00-1	(*S*)-(−)[b]	730-15-4	COOH	$CH_2CH(CH_3)_2$
N-PA-phenylalanine (5)	54582-05-7	(*S*)-(+)[b]	738-75-0	COOH	$CH_2C_6H_5$
N-PA-norvaline (6)	65415-01-2	(*S*)-(−)[c]	34337-08-1	COOH	$(CH_2)_2CH_3$
N-PA-phenylglycine (7)	35039-72-6	(*S*)-(+)[c]	24003-71-2	COOH	C_6H_5
N-PA-serine (8)	2752-41-2	(*S*)-(+)[b]	2752-53-6	COOH	CH_2OH
N-PA-aspartic acid (9)	17079-41-3	(*S*)-(+)[b]	2752-32-1	COOH	CH_2COOH
N-PA-glutamic acid (10)	2752-35-4	(*S*)-(−)[b]	2752-33-2	COOH	$(CH_2)_2COOH$
N-PA-alaninamide (11)	65415-02-3	(*S*)-(−)[c]	65451-29-8	$CONH_2$	CH_3
N-PA-2-aminobutyramide (12)	65415-03-4	(*S*)-(−)[c]	65451-30-1	$CONH_2$	C_2H_5
N-PA-alaninonitrile (13)	31962-96-6	(*S*)-(−)[d]	65451-31-2	CN	CH_3
N-PA-2-aminobutyronitrile (14)	34096-57-6	(*S*)-(−)[d]	65451-32-3	CN	C_2H_5
N-PA-valinonitrile (15)	31962-95-5	(*R*)-(+)[d]	65451-33-4	$CH(CH_3)_2$	CN
N-PA-alaninol (16)	34084-11-2	(*S*)-(−)[d]	65451-13-0	CH_2OH	CH_3
N-PA-2-aminobutanol (17)	34114-57-3	(*S*)-(−)[d]	65451-14-1	CH_2OH	C_2H_5
N-PA-3-aminobutyric acid (18)	65451-17-4	(*S*)-(−)[e]	65414-81-5	CH_2COOH	CH_3
N-PA-3-aminovaleric acid (19)	65451-18-5	(*S*)-(−)[e]	65414-82-6	CH_2COOH	C_2H_5
N-PA-3-amino-3-phenylpropionic acid (20)	65451-19-6	(*S*)-(+)[e]	65414-83-7	CH_2COOH	C_6H_5
N-PA-4-aminovaleric acid (21)	65451-20-9	(*S*)-(−)[e]	65414-84-8	$(CH_2)_2COOH$	CH_3
N-PA-5-aminohexanoic acid (22)	65451-21-0	(*S*)-(−)[e]	65414-85-9	$(CH_2)_3COOH$	CH_3
N-PA-6-aminoheptanoic acid (23)	65451-22-1	(*S*)-(−)[e]	65414-86-0	$(CH_2)_4COOH$	CH_3
N-PA-2-aminobutane (24)	56649-69-5	(*S*)-(+)[f]	56572-16-8	C_2H_5	CH_3
N-PA-*p*-hydroxyamphetamine (25)	34096-54-3	(*S*)-(−)[d]	65451-15-2	$CH_2C_6H_4$-*p*-OH	CH_3
N-PA-norleucinonitrile (26)	65451-23-2	(*S*)-(−)[f]	65414-87-1	CN	$(CH_2)_3CH_3$
N-PA-leucinonitrile (27)	65451-24-3	(*S*)-(−)[f]	65414-88-2	CN	$CH_2CH(CH_3)_2$
N-PA-valinol (28)	65451-25-4	(*S*)-(−)[f]	65414-89-3	CH_2OH	$CH(CH_3)_2$
N-PA-serinonitrile (29)	65451-26-5	(*S*)-(+)[f,g]	65414-90-6	CH_2OH	CN
N-PA-serinamide (30)	27826-53-5	(*S*)-(+)[f]	27820-89-9	$CONH_2$	CH_2OH
N-PA-serine methyl ester (31)	65415-04-5	(*S*)-(−)[f]	65414-91-7	$COOCH_3$	CH_2OH
4-Cyano-4-phenylacetamidobutyric acid (32)	65451-27-6	(*R*)-(+)[f]	65414-92-8	$(CH_2)_2COOH$	CN
Methyl 4-cyano-4-phenylacetamidobutyrate (33)	65451-28-7	(*R*)-(+)[f]	65414-93-9	$(CH_2)_2COOCH_3$	CN
N-PA-aspartic acid α-methyl ester (34)	65415-05-6	(*S*)-(−)[f]	65414-63-3	$COOCH_3$	CH_2COOH
N-PA-glutamic acid α-methyl ester (35)	65415-06-7	(*S*)-(−)[f]	65414-94-0	$COOCH_3$	$(CH_2)_2COOH$

[a] The sign of the optical rotations is referred to the sodium D line and methanol or ethanol as solvent. [b] T. Suyama, T. Toyoda, and S. Kanao, *Yakugaku Zasshi*, **85**, 279 (1965). [c] Specific rotation has been determined in the course of the present work since previously not reported: **2**, $[\alpha]^{20}_D$ −20.0° (*c* 4.5, ethanol); **6**, $[\alpha]^{20}_D$ −17.5° (*c* 4.0, ethanol); **7**, $[\alpha]^{20}_D$ +143° (*c* 1.0, ethanol); **11**, $[\alpha]^{20}_D$ −27.8° (*c* 4.0, methanol; **12**, $[\alpha]^{20}_D$ −28.3° (*c* 2.0, methanol). [d] See ref 5. [e] See ref 4. [f] See Table II. [g] In relation to the use of *R*/*S* and D/L nomenclatures, notice that for all the compounds in the table, the *S* configuration corresponds to L except for compound **29**. [h] PA = phenylacetyl.

preferred by the enzyme is known, the A and B position can be assigned to each pair of substituents (Table I). By examining A and B positions relative to the different substrates, substituents can be classified according to the decreasing tendency to occupy position A, i.e., priority sequences A > B can be defined. Positions A and B found for the substituents of each compound are reported in Table I. Analysis of these results, by using internal comparisons,[8] allowed three priority sequences to be defined.

By examining the results relative to *N*-phenylacetylvalinonitrile (**15**), to *N*-phenylacetylbutyronitrile (**14**), and to *N*-phenylacetyl-2-aminobutane (**24**), respectively, the relations $(CH_3)_2CH > CN$, $CN > C_2H_5$, and $C_2H_5 > CH_3$ can be obtained; see sequence I. One of the three possible priority relations between the noncontiguous terms of sequence I can be verified by examining hydrolytic data for *N*-phenylacetylalaninonitrile (**13**); the $CN > CH_3$ relation is found, as indicated in sequence I:

$$CH(CH_3)_2 > CN > C_2H_5 > CH_3$$

By examining hydrolytic results of substrates **8**, **31**, **30**; **28**; **15**; **13**, **14**, **26**, and **27** sequence II can be defined:

$$\left.\begin{matrix}COOH\\COOCH_3\\CONH_2\end{matrix}\right\} > CH_2OH > CH(CH_3)_2 > CN$$

$$> \left\{\begin{matrix}CH_2CH_3 > CH_3\\CH_2CH(CH_3)_2\\CH_2(CH_2)_2CH_3\end{matrix}\right.$$

From the hydrolysis data of compounds **9**, **10**, **34**, **35**; **32** and **33** sequence III can be analogously defined. Hydrolysis data

$$\left.\begin{matrix}COOH\\COOCH_3\end{matrix}\right\} > \left\{\begin{matrix}CH_2COOH\\CH_2CH_2COOH\\CH_2CH_2COOCH_3\end{matrix}\right\} > CN$$

Table II. Hydrolytic Data Relative to *N*-Phenylacetylamino Compounds[a]

Substrate No.	mg	Buffer, mL	Enzyme, mg	Incubn time, h	Hydrolysis, %	$[\alpha]_D$, deg,[b] of substrates: Recovered	S isomers
(±)-**26**	200	370[c]	0.50	20	70	+30 (*c* 4.0)	
(±)-**27**	200	370[c]	0.50	20	42	+33 (*c* 4.0)	
(±)-**28**	150	30[c]	3.50	240[d]	49	+5.8 (*c* 6.0)	−30 (*c* 3.0)
(±)-**29**	600	120[c]	0.70	5	57	−2.1 (*c* 7.0)	
(±)-**30**	300	60[c]	0.50	24	50	−3.0 (*c* 7.0)	+4.0 (*c* 4.0)
(±)-**31**[e]	150	40[c,f]	1.10	24	66	+11 (*c* 1.5)	−13 (*c* 1.5)
(±)-**32**	500	100	0.35	18	75	−12 (*c* 2.0)	
(±)-**33**	200	50[c,f]	0.20	24	46	−1.4 (*c* 6.0)	−15.7 (*c* 2.0)[g]
(±)-**9**[h]	300	70[f]	0.30	20	48	−9.0 (*c* 5.0)[i]	+10.4 (*c* 2.0)[i]
(±)-**34**	600	150[f]	0.60	14	52	+3.0 (*c* 5.0)	−14.5 (*c* 4.0)
(*S*)-**34**	600	150[f]	0.60	18	70		
(*R*)-**34**	600	150[f]	0.60	18	65		
(±)-**35**	500	100[f]	0.60	24	47	+19 (*c* 2.0)	−31.5 (*c* 1.0)
(*S*)-**35**	850	170[f]	0.85	16	65		
(*R*)-**35**	500	100[f]	0.50	20	10		
(±)-**16**[h]	150	20	0.50	24[d]	52	+12 (*c* 4.0)	−15.2 (*c* 3.0)
(±)-**17**[h]	150	30	1.00	24[d]	50	+9.2 (*c* 4.0)	−34 (*c* 4.0)
(±)-**24**[h]	200	45	2.00	24[d]	53	−9.2 (*c* 3.0)	+17 (*c* 3.0)

[a] Hydrolysis experiments were carried out in 0.1 M phosphate buffer, pH 7.0, and at a temperature of 30 °C unless otherwise indicated. [b] All values were determined in methanol at 20 °C unless otherwise specified. [c] Because of the low solubility of the substrate, methanol was added (8% v/v). [d] Incubation temperature 37 °C. [e] A. Romeo and G. Di Maio, *Ann. Chim. (Rome)*, **47**, 675 (1957). [f] pH 6.2. [g] Obtained as an oil by treating (−)-**32** with diazomethane. [h] Hydrolysis experiments on this substrate were previously reported. See ref 5 and 6. [i] Solvent ethanol.

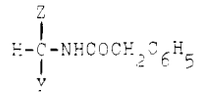
Table III. Substrates with Unknown Absolute Configuration. Configurational Correlation by Chemical Methods

Compd[a]	$[\alpha]^{20}_D$, deg[b]: Reaction product	*S* isomer
N-Phenylacetylnorleucine methyl ester (**36**) from (+)-**26**	+16.3 (*c* 1.0)	−31 (*c* 4.0)
N-Phenylacetylleucine methyl ester (**37**) from (+)-**27**	+3.3 (*c* 4.0)	−41 (*c* 4.0)[c]
N-Phenylacetylserinamide (**30**)[d] from (−)-**29**	$\Delta\epsilon$ −0.1 (220 nm)	$\Delta\epsilon$ −1.4 (220 nm)
Glutamic acid from (−)-**32**	+26.4 (*c* 1.5, 5 N HCl)	+31 (*c* 1.5, 5 N HCl)

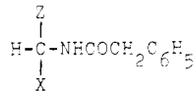
[a] Compounds into which the recovered substrates (+)-**26**, (+)-**27**, (−)-**29**, and (−)-**32** were transformed (see Experimental Section). [b] Solvent methanol unless otherwise specified. [c] Lit. $[\alpha]^{20}_D$ −13° (*c* 2.0, ether); cf. H. T. Clarke, J. R. Johnson, and R. Robinson, "The Chemistry of Penicillin", Princeton University Press, Princeton, N.J., 1949, p 786. [d] Because of the low value of the $[\alpha]_D$, CD_{max} in methanol are reported.

of *N*-phenylacetylserinonitrile (**29**), of *N*-phenylacetylamino alcohols **16** and **17**, of *N*-phenylacetylamino amides **11** and **12**, and of the *N*-phenylacetylamino acids are in accordance with sequence II which was defined (as for sequence I) by using the indicated internal comparisons between contiguous terms.

In order to further analyze priority relations and to better define sequences II and III, hydrolytic results have been examined by making use of external comparisons.[8] The following general procedure was adopted; a priority relation between two substituents Y and X was established by comparing the stereoselectivity of the hydrolysis of two substrates, i and ii,



i



ii

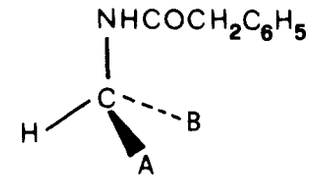


Figure 1. Configuration of the more rapidly hydrolyzed enantiomer.

containing the common substituent Z. Two cases have been considered.

(1) The Z group is in position A with respect to the two groups under examination [Z > (Y; X)]. Priority is then assigned to that substituent (Y or X) which confers lower stereoselectivity to the hydrolysis (e.g., Y > X if the hydrolysis of i is found to be less stereoselective than that of ii).

(2) The two groups Y and X are both in position A with respect to the common group [(Y; X) > Z]. Priority is assigned to that substituent (Y or X) which confers higher stereoselectivity to the hydrolysis (e.g., Y > X if the hydrolysis of i is more selective than that of ii).

Priority relations deduced by using the above described external comparisons are given in Table IV. The data show that for all the substrates examined, the relations obtained through external comparison agree with the relations already defined by using internal comparison. A direct confirmation of this accordance can be found by comparing the relations $C_2H_5 > CH_3$ and $CH_2OH > CH_3$, given in Table IV, with the corresponding ones based on the hydrolysis of *N*-phenylacetyl-2-aminobutane (**24**) and of *N*-phenylacetylalaninol (**16**), respectively. The relation $CH_2COOH > CH_2CH_2COOH$ was confirmed by performing separate hydrolysis experiments (Table II) on the two enantiomers of *N*-phenylacetylaspartic acid α-methyl ester, (*S*)-**34** and (*R*)-**34**, and on those of *N*-phenylacetylglutamic acid α-methyl ester, (*S*)-**35** and (*R*)-**35**. In accordance with the assigned priority, the data given in Table II show that the difference between the hydrolysis rates of the two enantiomers is clearly lower for **34** than for **35**. The following relations, $COOH > COOCH_3 > CONH_2$ and $CH_2COOH > CH_2CH_2COOH$, found by external comparison, can then be introduced into sequences II and III, respectively, and some of the unresolved priorities can be defined.

Table IV. Priority Relations Based on External Comparisons

Compd	Positions of substituents in model of Figure 1 A	B	Optical purity, %[a]	Priority relation
16	CH_2OH	CH_3	79	$C_2H_5 > CH_3$
17	CH_2OH	CH_2CH_3	27	
28	CH_2OH	$CH(CH_3)_2$	19	$CH(CH_3)_2 > CH_3$
16	CH_2OH	CH_3	79	
28	CH_2OH	$CH(CH_3)_2$	19	$CH(CH_3)_2 > C_2H_5$
17	CH_2OH	CH_2CH_3	27	
16	CH_2OH	CH_3	79	$CH_2OH > CH_3$
24	CH_2CH_3	CH_3	54	
9	COOH	CH_2COOH	87	$COOH > COOCH_3$
34	$COOCH_3$	CH_2COOH	21	
35	$COOCH_3$	$(CH_2)_2COOH$	60	$CH_2COOH > (CH_2)_2COOH$
34	$COOCH_3$	CH_2COOH	21	
30	$CONH_2$	CH_2OH	75	$COOCH_3 > CONH_2$
31	$COOCH_3$	CH_2OH	85	

[a] $100 \times [\alpha]_D$ obsd/$[\alpha]_D$ max.

The priority relations found in the present investigation clearly indicate that relative arrangement of the substituents in the A > B sequence is controlled by different factors whose contribution is, at the present, difficult to evaluate. In the series of the *N*-phenylacetyl-α-amino acids, the COOH group is found in position A for all the substrates examined and a progressive decrease of the hydrolysis rate[3] is observed as the size of the substituents at the chiral center increases. On the other hand, data relative to the hydrolysis of the other series of *N*-phenylacetylamino compounds lead to priority relations which are, in some cases, anomalous in terms of the usual concepts of the sizes of the groups concerned, e.g., $CN > C_2H_5$, $CH_2OH > CH(CH_3)_2$, $CN > CH_2CH(CH_3)_2$. In this context, the inversion of the steric course of the hydrolysis found on passing from *N*-phenylacetylalaninonitrile (**13**) and *N*-phenylacetyl-2-aminobutyronitrile (**14**) to *N*-valinonitrile (**15**) and *N*-phenylacetylserinonitrile (**29**) is of note. Steric hindrance as well as polarity of the substituents is then to be considered among the most effective factor regulating the steric course of the hydrolysis.

Given the absence of information on the active site, the data reported here constitute a contribution to elaborate empirical guidelines for stereochemical predictions in the BPA-catalyzed hydrolyses of the amide bond. Further studies are in progress to unify the proposed priority sequences and to extend the analysis to new substituents.

Experimental Section

General. Melting points were determined in capillary tubes and are uncorrected. Preparative layer chromatography (PLC) was carried out with Merck HF_{254} silica gel on 0.5 mm thick plates. Optical rotations were taken at 20 °C with a Schmidt & Haensch 16065 polarimeter. IR spectra were recorded on a Perkin-Elmer 521 spectrophotometer. The mass spectrum was determined with an A.E.I. MS 12 spectrometer.

Enzyme. Hydrolysis experiments were performed using BPA prepared as follows. *Escherichia coli* cells were grown at 24 °C for 24 h in a medium containing 1.0% peptone, 1.0% meat extract (Acas, A. Costantino, Italy), 0.5% NaCl, and 0.2% phenylacetic acid. The pH was adjusted to 7.0 with sodium hydroxide. Cells were separated from the medium by centrifugation, disintegrated with the X-Press (AB Biox, Sweden), and extracted with 0.3 M phosphate buffer, pH 7.0. After centrifugation, 30% v/v of 1% protamine sulfate in 0.1 M phosphate buffer, pH 7.0, was added to the solution, and nucleic acids were removed by centrifugation. Cold ammonium sulfate was then added to the supernatant, and proteins precipitating between 30 and 60% saturation were collected. The precipitate was dissolved with 0.02 M phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer. The protein solution was then fractionated on a DEAE-Sephadex A50 column equilibrated with the same buffer. Fractions with highest hydrolytic activity were collected and concentrated on a UM10 DIAFLO membrane (Amicon, USA). This solution contained 30 mg of protein/mL with a specific activity of 2000 units[9] per milligram; further steps of fractionation did not result in significant improvement of this value.

Substrate Recovery. General Procedure. Hydrolysis experiments were carried out in 0.1 M phosphate buffer at the pH and temperature reported in Table II. The time course of production of phenylacetic acid was followed by gas chromatography (internal standard methyl benzoate) by esterifying with diazomethane aliquots at suitable intervals. Neutral *N*-phenylacetyl derivatives were separated from the phenylacetic acid and from the amino compounds released by the enzyme, following usual fractionation with solvents. Acidic *N*-phenylacetyl derivatives were separated from phenylacetic acid by column chromatography on silica gel (3 g of silica per 100 mg of residue). Elution with benzene–ethyl acetate (8:2) gave phenylacetic acid. On subsequent elution with ethyl acetate, the unreacted *N*-phenylacetyl derivative was recovered in practically quantitative yield.

(±)-*N*-Phenylacetylnorleucinonitrile (26). This was obtained by acylating the corresponding aminonitrile[10] with phenylacetyl chloride in aqueous sodium bicarbonate solution containing 20% tetrahydrofuran: mp 71–72 °C from ether–petroleum ether (65% yield). Anal. Calcd for $C_{14}H_{18}N_2O$: C, 73.01; H, 7.88; N, 12.16. Found: C, 72.97; H, 7.90; N, 12.07.

(±)-*N*-Phenylacetylleucinonitrile (27). The procedure adopted for **26** was followed. Leucinonitrile was prepared according to the literature.[10] **27** was crystallized from ethyl acetate–petroleum ether: mp 96 °C (60% yield). Anal. Calcd for $C_{14}H_{18}N_2O$: C, 73.01; H, 7.88; N, 12.16. Found: C, 72.92; H, 7.97; N, 12.17.

(±)-*N*-Phenylacetylvalinol (28). Phenylacetyl chloride (2.79 g, 18.15 mmol) was added dropwise (1 h) at 0 °C to a stirred solution of 1.25 g (12.1 mmol) of (±)-valinol[11] in 10 mL of 20% sodium hydroxide. After an additional 0.5 h of stirring at room temperature the mixture was extracted with ether. The extract, washed with 0.5 N hydrochloric acid and water and dried, gave a residue which was crystallized from ether: 1.06 g (40%); mp 91–92 °C; IR ($CHCl_3$) 3410, 2940, 1645, 1590 cm^{-1}. Anal. Calcd for $C_{13}H_{19}NO_2$: C, 70.55; H, 8.65; N, 6.33. Found: C, 70.63; H, 8.58; N, 6.27.

From the mother liquors of the crystallization of **28** the *N*,*O*-diphenylacetyl derivative of the starting amino alcohol was isolated: mp 87–88 °C from ether. Anal. Calcd for $C_{21}H_{25}NO_3$: C, 74.31; H, 7.42; N, 4.13. Found: C, 74.40; H, 7.42; N, 4.07.

(*S*)-*N*-Phenylacetylvalinol [(*S*)-28] was prepared from (*S*)-valinol as described above for **28:** mp 72 °C from ether (37% yield);

$[\alpha]^{25}_D$ −30° (c 3.0, methanol). Anal. Calcd for $C_{13}H_{19}NO_2$: C, 70.55; H, 8.65; N, 6.33. Found: C, 70.55; H, 8.72; N, 6.35.

(±)-*N*-Phenylacetylserinonitrile (29). (2-Tetrahydropyranyloxy)acetaldehyde[12] (5.0 g, 34.7 mmol) in 15 mL of ether was saturated with ammonia at 10 °C. The solution was then evaporated and the residue carefully dried under vacuum to give *O*-(2-tetrahydropyranyl)-2-iminoethanol. To the crude imino derivative, 3.5 mL of dry hydrogen cyanide was added and the solution was allowed to stand overnight at 18 °C. After evaporation of the hydrogen cyanide the residue was dissolved in 120 mL of dry benzene containing 2.74 g of pyridine. Phenylacetyl chloride (5.34 g, 34.7 mmol) in 50 mL of dry benzene was added under stirring, during a 20-min period, at room temperature. After an additional 15 h of stirring, the solution was washed with cooled 1 N hydrochloric acid (40 mL), aqueous sodium bicarbonate, and water. The organic layer was dried over Na_2SO_4 and evaporated to give 6.5 g of an oily residue. Purification by PLC (eluent, 1:1 ether–petroleum ether) gave 2.25 g (23%) of 2-phenylacetamido-3-(2-tetrahydropyranyloxy)propanenitrile. The mass spectrum taken at 70 eV gave a molecular ion peak at *m/e* 288; IR ($CHCl_3$) 3405, 2935, 2860, 2245, 1680, 1600, 1495 cm^{-1}. Anal. Calcd for $C_{16}H_{20}N_2O_3$: C, 66.65; H, 6.99; N, 9.72. Found: C, 66.35; H, 6.83; N, 9.48.

Hydrochloric acid (150 mL, 2 N) was added, at room temperature, to a stirred solution of 1.44 g (5.0 mmol) of the above product in 50 mL of methanol over a period of 15 min. After an additional 30 min of stirring, a slight excess of solid sodium bicarbonate was added. After removal of the methanol under vacuum at room temperature, the aqueous solution was extracted with ethyl acetate. Drying and evaporation of the solvent gave a residue which was crystallized from chloroform: 0.930 g of **29** (91%); mp 126–127 °C; IR ($CHCl_3$) 3405, 2925, 2245, 1670, 1600, 1485 cm^{-1}. Anal. Calcd for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.61; H, 5.93; N, 13.64.

***N*-Phenylacetyl-(±)-serinamide (30)** was obtained by treating (±)-serinamide hydrochloride in saturated aqueous sodium bicarbonate with phenylacetyl chloride at 0 °C: mp 144–145 °C (methanol–ethyl acetate); 37% yield. Anal. Calcd for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.38; H, 6.38; N, 12.47.

***N*-Phenylacetyl-(*S*)-serinamide [(*S*)-30]** was prepared from (*S*)-serinamide hydrochloride as described above for **30:** mp 137–138 °C (methanol–ethyl acetate); 30% yield; $[\alpha]^{20}_D$ + 4.0° (c 4.0, methanol). Anal. Calcd for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.49; H, 6.40; N, 12.51.

***N*-Phenylacetylserinamide (30) Starting from (−)-*N*-Phenylacetylserinonitrile (29).** The solid-phase catalysis of the hydrolysis of nitriles to amides reported by Cook[13] was utilized. Manganese dioxide (2 g) was added to a solution of 100 mg (0.49 mmol) of *N*-phenylacetylserinonitrile, $[\alpha]^{20}_D$ −2.0° (c 7.0, methanol), recovered from enzymatic hydrolysis of (±)-**29.** The mixture was stirred for 15 h at room temperature. The dioxide was filtered and repeatedly washed with 7:3 ethyl acetate–methanol. The combined solutions were evaporated under vacuum and the residue was purified by PLC (eluent, 93:7 ethyl acetate–methanol; 35 mg (32%) of *N*-phenylacetylserinamide was obtained. The optical data of this compound are reported in Table III.

(±)-4-Cyano-4-phenylacetamidobutyric Acid (32). 2-Ketoglutaric acid (21.9 g, 150 mmol) was added to a stirred solution containing 9.78 g (150 mmol) of KCN, 8.64 g (160 mmol) of NH_4Cl in 36 mL of water, and 36 mL of aqueous (25%) NH_3, over 20 min at 20 °C. The reaction mixture was allowed to stir for another hour at room temperature and for 4 h at 60 °C. NaOH (60 mL, 20%) was added to the cooled solution and the ammonia was removed under vacuum at room temperature. The aqueous alkaline solution was then treated, at 5 °C under stirring, with 30 g (195 mmol) of phenylacetyl chloride. During the acylation, portions of 20% NaOH were added to keep the pH at 8.5. The resulting solution was washed with ether and acidified (pH 2.5) at 5 °C with hydrochloric acid. The separated solid was crystallized from methanol–ethyl acetate to give 10.4 g (23%) of (±)-2-cyano-2-phenylacetamidoglutaric acid. The compound did not show a definite melting point but began to decompose, starting at 120 °C. Because of its thermal lability, this compound was analyzed as the dimethyl ester obtained by treatment with diazomethane in ether. The diester melted at 132–133 ° C (ethyl acetate–petroleum ether). Anal. Calcd for $C_{16}H_{18}N_2O_5$: C, 60.37; H, 5.70; N, 8.80. Found: C, 60.21; H, 5.71; N, 8.82.

(±)-2-Cyano-2-phenylacetamidoglutaric acid (9.0 g, 30.4 mmol) was heated under vacuum (0.1 mm) at 145 °C for 10 min. The glass obtained was purified by silica-gel (240 g) column chromatography. After washing the column with ethyl acetate, 30.2 g (43%) of **32** was eluted. The compound was crystallized from ethyl acetate–petroleum ether: mp 138–139 °C; IR (KBr) 3275, 2240, 1965, 1665 cm^{-1}. Anal. Calcd for $C_{13}H_{14}N_2O_3$: C, 63.40; H, 5.73; N, 11.38. Found: C, 63.16; H, 5.76; N, 11.21.

(±)-Methyl-4-cyano-4-phenylacetamidobutyrate (33). This compound was obtained by treating **32** with diazomethane in ether: mp 72–73 °C (ethyl acetate–petroleum ether); IR (KBr) 3260, 2240, 1725, 1650 cm^{-1}. Anal. Calcd for $C_{14}H_{16}N_2O_3$: C, 64.60; H, 6.20; N, 10.76. Found: C, 64.60; H, 6.22; N, 10.82.

Preparation of *N*-Phenylacetyl Derivatives of α-Methyl Esters of Aspartic and Glutamic Acids [(±)-34, (*S*)-34, (*R*)-34, (±)-35, (*S*)-35, and (*R*)-35]. The amino derivatives corresponding to the title compounds were prepared according to general procedure reported by Kovacs and co-workers.[14] The *N*-phenylacetyl derivatives were prepared according to the following procedure relative to (±)-**34.** Phenylacetyl chloride (680 mg, 4.4 mmol) was added dropwise (1 h) to a stirred solution of 285 mg (3.4 mmol) of $NaHCO_3$ and 500 mg (3.4 mmol) of (±)-aspartic acid α-methyl ester in 5 mL of water at 0 °C. The solution was carefully maintained at pH 7.5 by adding 1 N NaOH. After washing with ether, the solution was acidified and extracted with ethyl acetate. The oily residue (890 mg) was purified by column chromatography on silica gel (23 g). After elution of phenylacetic acid with 6:4 benzene–ethyl acetate, the *N*-phenylacetyl derivative (±)-**34** was eluted with 9:1 ethyl acetate–acetic acid: 785 mg (67%); mp 111–113 °C (ethyl acetate–petroleum ether). Anal. Calcd for $C_{13}H_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28. Found: C, 58.59; H, 5.80; N, 5.15.

***N*-Phenylacetyl-(*S*)-aspartic acid α-methyl ester [(*S*)-34]** was obtained as an oil (60% yield): $[\alpha]^{20}_D$ −14.5° (c 4.0, methanol). For elemental analysis the dicyclohexylamine salt was prepared: mp 165–166 °C (dry ethyl ether); $[\alpha]^{20}_D$ +5.8° (c 3.0, methanol). Anal. Calcd for $C_{25}H_{38}N_2O_5$: C, 67.23; H, 8.58; N, 6.27. Found: C, 67.32; H, 8.63; N, 6.29.

***N*-Phenylacetyl-(*R*)-aspartic acid α-methyl ester [(*R*)-34]** was obtained as an oil (70% yield): $[\alpha]^{20}_D$ +14.0° (c 4.0, methanol). For elemental analysis the dicyclohexylamine salt was prepared: mp 165 °C (dry ethyl ether); $[\alpha]^{20}_D$ −5.5 ° (c 3.0, methanol). Anal. Calcd for $C_{25}H_{38}N_2O_5$: C, 67.23; H, 8.58; N, 6.27. Found: C, 67.04; H, 8.71; N, 6.21.

***N*-Phenylacetyl-(±)-glutamic acid α-methyl ester [(±)-35]** was crystallized from ethyl acetate–petroleum ether (65% yield): mp 79–80 °C. Anal. Calcd for $C_{14}H_{17}NO_5$: C, 60.20; H, 6.14; N, 5.02. Found: C, 60.13; H, 6.05; N, 4.95.

***N*-Phenylacetyl-(*S*)-glutamic acid α-methyl ester [(*S*)-35]** was crystallized from ethyl acetate–petroleum ether (60% yield): mp 103–104 °C; $[\alpha]^{20}_D$ −31.5° (c 1.0, methanol). Anal. Calcd for $C_{14}H_{17}NO_5$: C, 60.20; H, 6.14; N, 5.02. Found: C, 60.10; H, 6.10; N, 4.93.

***N*-Phenylacetyl-(*R*)-glutamic acid α-methyl ester [(*R*)-35]** was crystallized from ethyl acetate–petroleum ether (65% yield): mp 103 °C; $[\alpha]^{20}_D$ +32.0° (c 1.0, methanol). Anal. Calcd for $C_{14}H_{17}NO_5$: C, 60.20; H, 6.14; N, 5.02. Found: C, 59.94; H, 6.20; N, 4.89.

***N*-Phenylacetyl-(*S*)-norleucine Methyl Ester [(*S*)-36].** (*S*)-Norleucine was treated in aqueous sodium hydroxide (20%) at 0 °C with phenylacetyl chloride. Usual workup gave a 55% yield of *N*-phenylacetyl-(*S*)-norleucine: mp 92–93 °C (ethyl acetate–hexane); $[\alpha]^{20}_D$ −12.0° (c 4.0, methanol). Anal. Calcd for $C_{14}H_{19}NO_3$: C, 67.45; H, 7.68; N, 5.62. Found: C, 67.38; H, 7.65; N, 5.58.

The *N*-phenylacetylamino acid was then treated with diazomethane in ether to give *N*-phenylacetyl-(*S*)-norleucine methyl ester: mp 56–57 °C (ethyl acetate–hexane); $[\alpha]^{20}_D$ −31.0° (c 4.0, methanol). Anal. Calcd for $C_{15}H_{21}NO_3$: C, 68.42; H, 8.04; N, 5.32. Found: C, 68.50; H, 8.06; N, 5.22.

***N*-Phenylacetylnorleucine Methyl Ester (36) Starting from (+)-*N*-Phenylacetylnorleucinonitrile (26).** A solution of 0.26 g (1.13 mmol) of *N*-phenylacetylnorleucinonitrile, $[\alpha]^{20}_D$ +30.0° (c 4.0, methanol), recovered from enzymatic hydrolysis of (±)-**26,** in 45 mL of dry methanol was saturated at room temperature with gaseous hydrogen chloride. The solution was refluxed for 1.5 h. After removal of the solvent, the residue was taken up with ether and the resulting solution washed with sodium bicarbonate solution and water. Usual workup gave an oily residue. PLC (eluent, 1:1 benzene–ether) afforded 50 mg of the compound: pure by TLC examination; the IR spectrum ($CHCl_3$) was identical with those of the above reported *S* isomer; $[\alpha]^{20}_D$ +16.3° (c 1.0, methanol).

***N*-Phenylacetylleucine Methyl Ester (37).** This was prepared from (+)-*N*-phenylacetylleucinonitrile, $[\alpha]^{20}_D$ +33.0° (c 4.0, methanol), recovered from the enzymatic hydrolysis of (±)-**27.** The method reported for the preceding compound was followed. A compound which resulted, identical with an authentic specimen of *N*-phenylacetylleucine methyl ester (see footnote c in Table III), was obtained (20% yield): $[\alpha]^{20}_D$ +3.3° (c 4.0, methanol).

Glutamic Acid from (−)-4-Cyano-4-phenylacetamidobutyric

Acid. This transformation (Table III) was performed by refluxing (−)-4-cyano-4-phenylacetamidobutyric acid, $[\alpha]^{20}_D$ −12.0° (c 2.0, methanol), recovered from the enzymatic hydrolysis of (±)-**32**, with 3 N hydrochloric acid.

Registry No.—(*R*)-**26**, 65414-95-1; (*R*)-**27**, 65414-96-2; **28** PA ester, 65414-97-3; (*R*)-**29**, 65414-98-4; **29** THP ether, 65414-99-5; (*S*)-**32**, 65414-60-0; (*R*)-**34**, 65414-61-1; (*R*)-**34** salt, 65414-62-2; (*S*)-**34** salt, 65414-64-4; (*R*)-**35**, 65414-65-5; (*S*)-**36**, 65414-66-6; (*R*)-**36**, 65414-67-7; (*R*)-**37**, 65414-68-8; norleucinonitrile, 65414-69-9; phenylacetyl chloride, 103-80-0; leucinonitrile, 65451-12-9; valinol, 16369-05-4; (*S*)-valinol, 22464-36-4; (2-tetrahydropyranyloxy)acetaldehyde, 65414-70-2; *O*-(2-tetrahydropyranyl)-2-iminoethanol, 65414-71-3; 2-amino-3-(2-tetrahydropyranyl)propionitrile, 65414-72-4; (±)-serinamide hydrochloride, 65414-73-5; (*S*)-serinamide hydrochloride, 65414-74-6; (±)-2-cyano-2-phenylacetamidoglutaric acid, 65414-75-7; (±)-2-cyano-2-phenylacetamidoglutaric acid dimethyl ester, 65414-76-8; diazomethane, 334-88-3; (±)-aspartic acid α-methyl ester, 65414-77-9; (*S*)-aspartic acid α-methyl ester, 17812-32-7; (*R*)-aspartic acid α-methyl ester, 65414-78-0; (±)-glutamic acid α-methyl ester, 65414-79-1; (*S*)-glutamic acid α-methyl ester, 6384-08-3; (*R*)-glutamic acid α-methyl ester, 26566-13-2; (*S*)-norleucine, 327-57-1; *N*-phenylacetyl-(*S*)-norleucine, 65414-80-4; benzylpenicillinacylase, 9014-06-6.

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Reactions of Protonated Diamino Acids in the Gas Phase

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The methane chemical ionization mass spectra of series of α,ω-diamino acids, ω-amino acids, cyclic and acyclic α-amino acids, and methyl esters have been obtained. Protonated α,ω-diamino acids react in the gas phase through the competitive cycloelimination of water or ammonia, decarboxylation, or collision stabilization of the intramolecularly hydrogen bonded protonated molecular ion. Structural factors which select between decarboxylation and lactam, lactone, and cyclic amino acid formation are determined by comparison of spectra of these related compounds. The prevalence of reactions correlates with the product ion stability and not with the site of protonation, the thermodynamically preferred site of protonation, or the stability of the intramolecularly hydrogen-bonded complex.

Reactions of protonated diamino acids in the gas phase may be studied under conditions of chemical ionization mass spectrometry. Under these conditions the diamino acid is protonated on a single site by way of an exothermic proton transfer reaction with reagent gas ions CH_5^+ or $CH_3CH_2^+$. The protonated molecular ions may undergo collision stabilization while in the ion source[1] or react through elimination of water, ammonia, or carbon monoxide.[2] In many ways these conditions are analogous to those in solution. The reactions of protonated molecules which occur in the gas phase but are not observed in solution demonstrate the influence of solvent effects on molecular reactivity.

Protonated molecules such as the diamino acids, 2,3-diaminopropionic to 2,6-diaminohexanoic acid (lysine), may react in the gas phase through simple S_E1 elimination analogous to reactions in strongly acid solution[3,4] or neighboring group displacement reactions involving three- to seven-membered cyclic transition states.[5-7] While gas phase reaction mechanisms may be analogous to solution chemistry, the charge on a protonated site is not distributed through solvation so that internal effects such as substituent polarizability,[6] hydrogen bonding,[8,9,10] and ion–dipole interactions[11,12,13] are relatively more important.

The reactions of protonated diamino acids are related to the interfunctional distance between the terminal amine and the α-amino acid moiety which indicates that neighboring group interactions may be involved. Lactam, lactone, and cyclic amino acid formation as well as decarboxylation reactions are believed to occur in the gas phase.

Our investigation of these reactions has centered on determining which intramolecular interactions (amine–amine or amine–carboxyl) are involved in diamino acid fragmentation and the structural features which regulate the probability of their occurrence. Although reaction product structures cannot be determined directly, supporting evidence may be obtained by comparing product ion reactivities to the reactivity of ions generated from other sources. For example, the subsequent fragmentation of the $MH-NH_3$ ion products, reactions 1–3, may be compared to protonated cyclic amino acid reactions.

The general features of 2,5-diaminopentanoic acid (ornithine) and 2,6-diaminohexanoic acid (lysine) methane chemical ionization mass spectra have been reported previously. Milne et al.[2] noted the selective initial elimination of ammonia from the 6 position of lysine, reaction 1. The cyclization mechanism postulated was supported by studies of diaminoalkanes,[5] $NH_2(CH_2)_nNH_2$, in which the probability of ammonia loss paralleled the rate of cyclization of $Br(CH_2)_nNH_2$ in solution.[14] An additional sequence leading to the cyclic iminium ion with loss of ammonia from the 2 position was also indicated.[2] Leclercq and Desiderio[15] noted the facile loss of water from ornithine and suggested that this

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