(30) C. I. Branden and I. Lindqvist, *Acta Chem. Scand.*, **14**, 726 (1960).
(31) R. H. Cox and M. G. Newton, *J. Amer. Chem. Soc.*, **94**, 4212 (1972); R. H. Cox, B. S. Campbell, and M. G. Newton, *J. Org. Chem.*, **37**, 1557 (1972); P. Haake, J. P. McNeal, and E. J. Goldsmith, *J. Amer. Chem. Soc.*, **90**, 715 (1968).
(32) See "International Tables for X-Ray Crystallography," Vol. III, Kynoch Press, Birmingham, England, 1962, p 276.
(33) P–O–C angles measured for several acyclic phosphate esters,[36,41-43] six-membered ring phosphate esters,[37,39,40] and the *exo* ester group in five-membered ring phosphate[10-12] are approximately 120°.
(34) ORTEP-II, A Fortran thermal-ellipsoid plot program for crystal structure illustrations: C. K. Johnson, Oak Ridge National Laboratory, Oak Ridge, Tenn. 37831.
(35) M. Halmann, Ed., "Chemical Analysis, Analytical Chemistry of Phosphorus Compounds," Vol. 37, Wiley-Interscience, New York, N.Y., 1972, pp 192–193, and references cited therein.
(36) M. Calleri and J. C. Speakman, *Acta Crystallogr.*, **17**, 1097 (1964).
(37) M. Ul-Haque, C. N. Caughlan, and W. L. Moats, *J. Org. Chem.*, **35**, 1446 (1970).
(38) C. L. Coulter, *J. Amer. Chem. Soc.*, **95**, 570 (1973).
(39) T. A. Beineke, *Acta Crystallogr., Sect. B*, **25**, 413 (1969).
(40) J. Rodgers, D. W. White, and J. G. Verkade, *J. Chem. Soc., A*, 77 (1971).
(41) J. Pletcher, M. Sax, and C. S. Yoo, *Acta Crystallogr. Sect. B*, **28**, 378 (1972).
(42) G. W. Svetich and C. N. Caughlan, *Acta Crystallogr.*, **19**, 645 (1965).
(43) M. Ul-Haque and C. N. Caughlan, *Acta Crystallogr., Sect. B*, **26**, 1528 (1970).

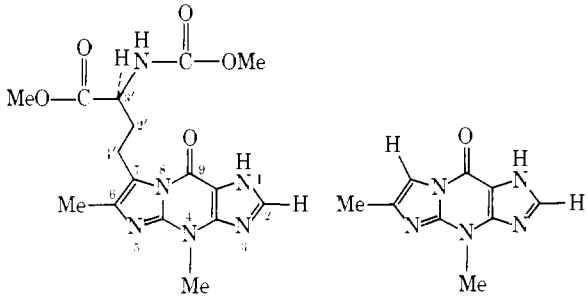
Isolation and Characterization of Peroxy-Y Base from Phenylalanine Transfer Ribonucleic Acid of the Plant, *Lupinus luteus*

A. M. Feinberg,[1a] K. Nakanishi,[1a]* J. Barciszewski,[1b] A. J. Rafalski,[1b] H. Augustyniak,[1b] and M. Wiewiórowski[1b]*

Contribution from the Chemistry Department, Columbia University, New York, New York 10027, and the Universytet im. Adama Mickiewicza, Instytut Chemii, Poznan, Grunwaldzka 6, Poland. *Received April 3, 1974*

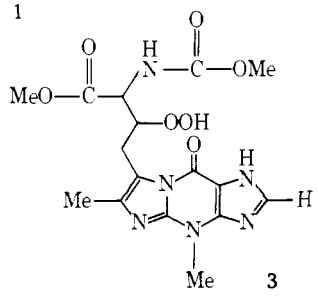
Abstract: The peroxy-Y structure **3** has been determined for the fluorescent base Y_L isolated from the phenylalanine tRNA of the plant *Lupinus luteus*. This compound had been shown to be present in the phenylalanine tRNA of beef, calf, chicken, and rat livers, but owing to the limited amount (*ca.* 5 μg from each source) it was only possible to measure the uv and mass spectra. The amount of material isolated from the plant (30 μg) provides additional evidence for this highly unique structure.

So far the following three hydrophobic fluorescent bases have been characterized from various sources of $tRNA^{Phe}$: Y_{sc} base **1** from Baker's yeast,[2,3] Y_t base **2** from *Torulopsis utilis*,[4] and peroxy-Y base **3** from livers of beef, calf, rat, and chicken.[5,6] The Y base first discovered in 1967, located next to the 3′ end of the anticodon loop,[7] can be selectively excised at pH 2.9.[8] It is intimately related to the codon-anticodon binding and has been shown by X-ray studies to be quite exposed (in the solid state of a salt).[9] Recently, guanine has been shown to be a biogenetic precursor of the Y base.[10]



1

2



3

Based on observations that the fluorescent bases present in wheat germ $tRNA^{Phe\ 11}$ and beef $tRNA^{Phe\ 5}$ are chromatographically and chemically similar, it was inferred that the wheat germ base could be represented by **3**.[6] The peroxy-Y base obtained from the various liver sources was very limited in amount (*ca.* 5 μg) of the base, so that the structure was derived solely from uv and mass spectra data. The plant *Lupinus luteus* (*Papilionaceae*) has provided a larger amount (30 μg) of the base and has thus enabled us to carry out CD measurements, and a microcolor reaction sensitive to peroxides. This has provided us with additional evidence supporting the very unique hydroperoxy structure **3**. As far as we know, this appears to be the first and only natural product characterized so far to have a hydroperoxy function.

Experimental Section

In view of the frequent difficulties encountered in the isolation of Y bases with satisfactory uv and fluorescent spectra, the isolation procedure is described in some detail. In this procedure, the base is excised directly from the crude tRNA mixture, and hence the laborious isolation of pure $tRNA^{Phe}$ is obviated.

Isolation of tRNA. All operations were performed at 5–8° in the cold room. The total tRNA mixture was isolated according to the following procedure. To a liter polyethylene container, 40 l. of freshly distilled phenol saturated with water was added, followed by 40 l. of distilled water containing 0.002 mol/l. of EDTA, 0.002 mol/l. of sodium thiosulfate, and 0.01 mol/l. of $MgCl_2$. The mixture was brought to pH 6.8–7.0 by addition of solid sodium acetate with stirring. Ten kilograms of freshly milled *Lupinus luteus* seeds was added with constant stirring, which was continued for 4 hr. The next day the separated upper phase was siphoned off to another polyethylene container. The lower phase was again stirred for 1 hr with 10 l. of 0.01 *M* $MgCl_2$, and the separated upper phase was also siphoned off to the same second container.

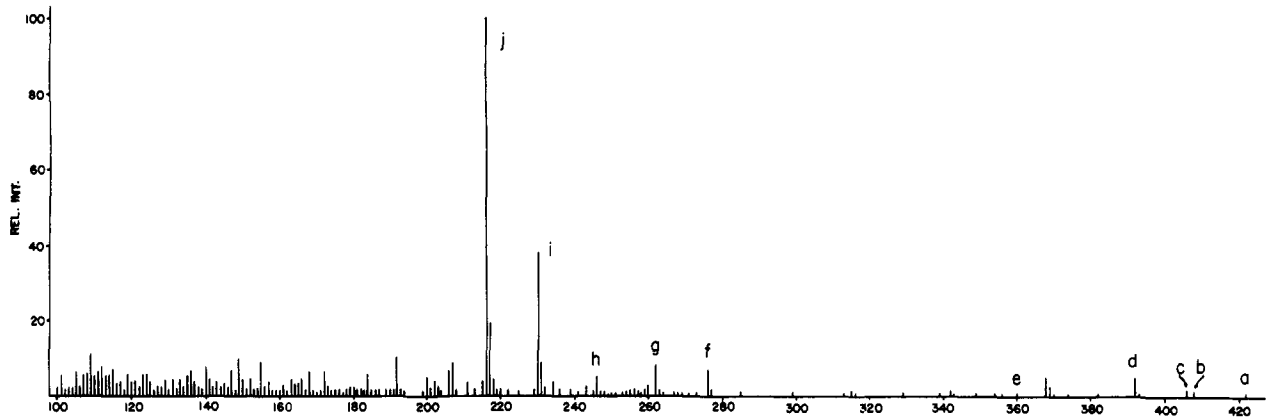


Figure 1. Mass spectrum of peroxy-Y base, MS-9, probe temperature 210°, 70 eV, PFK standard, $m/\Delta m$ = 10,000. Alphabets denote peaks listed in Table I.

To the combined supernatants (upper phase) 0.25 volume of water saturated phenol was added, and the mixture was stirred for 2 hr. When the phases separated, the upper layer was siphoned off and subjected to further purification, as follows. Solid sodium acetate was added to final concentration of 0.3 mol/l., pH 6.5–6.6. A 0.25 volume of cold isopropyl alcohol was added with constant vigorous stirring. The precipitate which formed was centrifuged off and discarded. To the supernatant 2 volumes of cold ethanol was added to precipitate the tRNA.

The tRNA was collected by centrifugation and dissolved in 4 l. of 0.01 *M* Tris buffer, pH 7.5, containing 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, and 0.002 *M* $Na_2S_2O_3$. Four liters of a mixture of chloroform:isoamyl alcohol 24:1 (v/v) was added, and the suspension was shaken several times vigorously. The upper aqueous layer was separated and extracted again with the same mixture. The tRNA was precipitated from the aqueous phase by the addition of 2 vol of cold ethanol and 0.1 vol of 20% potassium acetate, pH 5.2. This step was repeated, using 2 l. of the Tris buffer and 2 l. of the chloroform mixture.

The precipitated crude tRNA was purified from a DEAE-cellulose column which had been throughly washed, according to standard procedures. After this step, the tRNA accepted *ca.* 100 pmol of phenylalanine/A_{260} unit in a standard enzymatic assay, using crude enzyme from *L. luteus*. This was the material used for the isolation of the fluorescent bases.

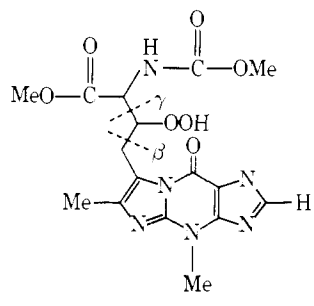
Occasionally this material was further purifed into two fractions by elution from another DEAE-cellulose column with a 0.3–0.65 *M* NaCl gradient.[12] The part of the tRNA (about one-half of the total) which was eluted with the high salt concentration contained most of the phenylalanine activity. Its specific activity was *ca.* 150 pmol Phe/A_{260} unit.

Isolation of the Fluorescent Bases from Total tRNA. The total crude tRNA (500 mg) was dissolved in 5 ml of H_2O and centrifuged. The supernatant was extracted 12 times with twice freshly distilled chloroform, and the water layer was centrifuged to remove an interphase precipitate. Ethanol (25 ml) was added and the solution kept at −70° for 2 hr to precipitate the tRNA. After centrifugation and complete removal of the ethanol, a white pellet remained. Five milliliters of phosphate buffer, pH 2.6, was added which when stirred gave a clear solution of pH 3.7. This was further acidified to pH 2.9 with 0.1 *N* HCl upon which a heavy precipitate formed. The cloudy solution was heated to 37° for 2 hr, stirred overnight at room temperature (20°), reheated to 37° for 4.5 hr more, and then neutralized with 0.1 *N* NaOH to pH 6.8 whereupon it cleared. The solution was then extracted six times with doubly distilled ethyl acetate, and the solvent was removed under reduced pressure at 15°. The residue was spotted on a tlc plate (which had been twice prewashed with developing solvent) and developed with ethyl acetate:1-propanol:H_2O (4:1:2) (upper layer). There appeared a major spot at R_F 0.54 and a minor spot (<10%) at R_F 0.66. Elution with ethyl acetate:1-propanol (5:1) gave *ca.* 18 μg and 1–2 μg (from uv) respectively of the major and minor products, the R_F values of which were identical in different solvent systems with the calf liver peroxy-Y base[6] and synthetic Y base.[3]

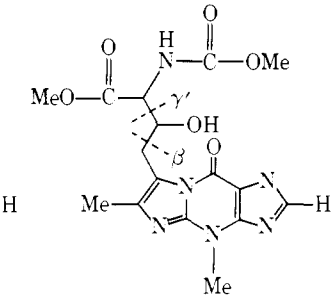
Results and Discussion

The chromatographic behavior of the present $tRNA^{Phe}$ on BD-cellulose gave the first suggestion that it was different from the yeast $tRNA^{Phe}$. Namely, although yeast $tRNA^{Phe}$ was only eluted with ethanol, *Lupinus luteus* $tRNA^{Phe}$ was eluted with salt, a behavior similar to that of liver $tRNA^{Phe}$.[6] Furthermore, addition of buffers containing 0.001 *M* sodium azide now caused *L. luteus* $tRNA^{Phe}$ to be eluted in the ethanol region, as was the case with liver $tRNA^{Phe}$.[6]

The mass spectrum, fluorescence, and uv data of the major component isolated from the final tlc purification were similar to those reported previously for peroxy-Y base.[6] The mass spectrum data obtained at 70 eV and a probe temperature of 210° are shown in Figure 1 and Table I (structures **4** and **5**). Pertinent fragmentations for the Y_{sc}



4



5

base are shown in structure **6** for the sake of comparison. High resolution values obtained for **4**, **5** + 14, and **5** were 408.1390 (calcd 408.1393), 406.1594 (calcd 406.1597), and 392.1437 (calcd 392.1444), respectively. A peak almost always present in natural Y base samples corresponded to a molecular weight of 368.3450 or $C_{27}H_{44}$ and hence appears to be a hydrocarbon impurity (perhaps dehydrocholesterol).

We had previously assigned[2] a series of +14 peaks as originating from addition of the solvent methanol followed by dehydration at the nuclear carbonyl group; accordingly these "artifact" +14 peaks were not discussed in the subsequent papers on peroxy-Y base, although they were present in the mass spectrum of those measured samples.[5,6] However, it was subsequently found that the peaks were still present, although at much reduced intensities, in samples of natural and synthetic Y_{sc} base and in natural peroxy-Y base which had never been in contact with methanol. Hence it is likely that an additional mechanism of methyl transfer is operating. It had been recorded earlier in the voacamine series[13] that when a methylating group such as a methoxycarbonyl group and an alkylatable group such as basic nitrogen

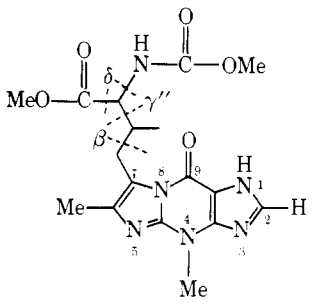
Table I. Pertinent Ions Observed in the Mass Spectrum of Peroxy-Y Base

Peaks	Ion[a]	Elemental composition	m/e[b]	Rel abundance, %
a	**4** + 14	$C_{17}H_{22}N_6O_7$	422	0.7
b	**4**	$C_{16}H_{20}N_6O_7$	408	1.6
c	**5** + 14	$C_{17}H_{22}N_6O_6$	406	1.9
d	**5** [**4** − (O)]	$C_{16}H_{20}N_6O_6$	392	5.0
e	**5** − MeOH	$C_{15}H_{16}N_6O_5$	360	0.7
f	γ + 14	$C_{12}H_{14}N_5O_3$	276	7.0
g	γ	$C_{11}H_{12}N_5O_3$	262	8.0
h	γ'	$C_{11}H_{12}N_5O_2$	246	5.0
i	β + 14	$C_{11}H_{12}N_5O$	230	38.0
j	β	$C_{10}H_{10}N_5O$	216	100.0

[a] Ions correspond to those indicated in structures **4** and **5** and Figure 1. [b] The measurements were on MS-9, 70 eV, probe temperature 210°, PFK standard, $m/\Delta m$ = 10,000.

are present in a molecule of low volatility, an intermolecular methyl transfer may take place. The Y bases with carbamate side chains fulfill this condition.[14] It was noticed that intensities of the +14 *m/e* peaks increased with higher probe temperature.

The loss of 16 mu's is one of the characteristics for the hydroperoxide group,[15] and this is the case in the present peroxy Y-base. Namely, two sets of fragmentation peaks are present, one arising from **4** and the other from **5.** The β-cleavage peak *m/e* 216 is present in both peroxy-Y **4** and Y_{sc} **6.** There is a two-oxygen difference in the γ-cleavage peaks of **4** and **6,** and hence the carbon β from the nucleus has to carry either a hydroperoxy group or has to be a ketal; the latter possibility is discounted on the grounds of loss of 16 mu's and not 17 (for loss of OH)[6] and also the color reactions described below.



6

M^+ + 14 (390) 1%
M^+ (376) 10%
M − MeOH (344) 1%
δ (242) 1%
γ'' + 14 (244) 4%
γ'' (230) 11%
β (216) 100%

The fluorescence maximum at 433 nm (excitation at 310) and the excitation maxima at 247, 276, and 322 nm (emission at 430) (in water) were similar to previous values.[6] As summarized in Table II and Figure 2, the CD

Table II. CD Data, nm ($\Delta\epsilon$) (Figure 2)

Peroxy Y (in H_2O):	204 (+1.24), 222 (−1.33), 239 (−2.34), 287 (−0.45)
Y (10% MeOH):	235 (−2.2), 264 (−0.36)

curve ($\Delta\epsilon$ values estimated from uv ϵ) was different from that of Y base reported earlier.[2] Although it is likely that

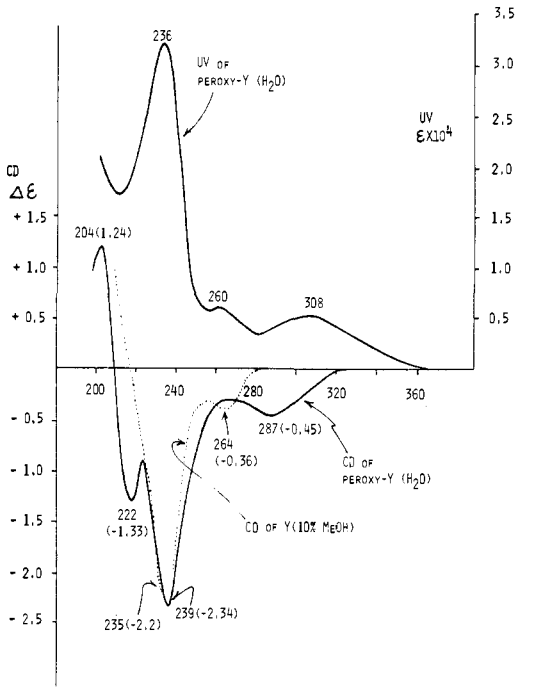


Figure 2. Uv and CD of peroxy-Y base in water and CD of Y base in 10% aqueous methanol.

the difference in the shorter wavelength CD extrema is due to the present better instrumentation, the difference in the longest wavelength CD minima should be noted (in spite of one being measured in water and the other in methanol). The peroxy-Y 287-nm minima may be due to two overlapping CD minima corresponding to the two uv maxima at 263 and 310 nm; the Y base 260-nm extremum corresponds to its 264-nm uv, while the CD peak corresponding to the 313-nm uv band could have been too weak to be measured.

Although the configuration at the chiral center of Y base **1** has been established to be S,[3] the present peroxy-Y **3** CD data are not sufficient for configurational deductions. Nevertheless, it clearly shows that perturbations of the aromatic nucleus caused by the chiral center(s) in the side chains of Y base **1** and peroxy-Y base **3** are different.

A FT nmr on the 30-μg sample only allowed the detection of four methyl peaks at 2.20, 3.71, 3.76, and 3.94 ppm. This compares favorably with the values obtained for **1,** namely, 2.26, 3.68, 3.71, and 3.96 ppm. The detection of other protons, however, was not possible, because the peaks were overlaid by absorptions due to contaminants collected from the silica gel of tlc plates.

The presence of the very rare hydroperoxide group was supported by a specific color test[16] employing ferrous thiocyanate solution. Namely, a pink red spot developed when an ethyl acetate solution of peroxy-Y base (a few micrograms) was applied to a filter paper or silica gel tlc plate sprayed with the reagent. Prior application of the base to filter paper or silica gel tlc plate followed by spraying with the ferrous thiocyanate reagent gave the same results. Parallel tests with synthetic Y base were negative; in contrast, the beef liver peroxy-Y base, isolated a year ago, responded positively, and this shows the remarkable stability of this unusual grouping. The minor fluorescent congener is most likely Y base **1** as judged from the R_F values and uv, although the limited amount (1 μg) failed to give a mass spectrum. The Y base **1** might be a biosynthetic precursor for the peroxy base **3,** but this point remains to be clarified.

The question of whether peroxy-Y base is an artifact formed during isolation was discussed previously.[6] Based on

previous evidence and the fact that in the present case it was by far the major fluorescent component of plant $tRNA^{Phe}$, we believe it to be a real constituent of $tRNA^{Phe}$. This is the first peroxy-Y base isolated from a higher plant source to have its structure determined.

Acknowledgments. We wish to thank Dr. George van Lear, Lederle Laboratories, for measurement of high-resolution mass spectra. This work has been supported by NIH Grant No. CA-11572, Hoffmann-La Roche Company (Columbia University), and Project 09.3.1 of the Polish Academy of Sciences.

References and Notes

(1) (a) Columbia University; (b) Universytet im. Adama Mickiewicza.
(2) K. Nakanishi, N. Furutachi, M. Funamizu, D. Grunberger, and I. B. Weinstein, *J. Amer. Chem. Soc.*, **92**, 7617 (1970).
(3) M. Funamizu, A. Terahara, A. Feinberg, and K. Nakanishi, *J. Amer. Chem. Soc.*, **93**, 6706 (1971).
(4) H. Kasai, M. Goto, S. Takemura, T. Goto, and S. Matsaura, *Tetrahedron Lett.*, 2725 (1971).
(5) K. Nakanishi, S. H. Blobstein, M. Funamizu, N. Furutachi, G. van Lear, D. Grunberger, K. Lanks, and I. B. Weinstein, *Nature (London)*, **234**, 107 (1971).
(6) S. H. Blobstein, D. Grunberger, I. B. Weinstein, and K. Nakanishi, *Biochemistry*, **12**, 188 (1973).
(7) U. L. RajBhandary, S. H. Chang, A. Stuart, R. C. Faulkner, R. M. Hoskinson, and H. G. Khorana, *Proc. Nat. Acad. Sci. U.S.*, **57**, 751 (1967).
(8) R. Thiebe and H. G. Zachau, *Eur. J. Biochem.*, **5**, 546 (1968).
(9) F. L. Suddath, G. J. Quigley, A. McPherson, D. Sneden, J. J. Kim, S. H. Kim, and A. Rich, *Nature (London)*, **248**, 20 (1974).
(10) H. J. Li, K. Nakanishi, D. Grunberger, and J. B. Weinstein, *Biochem. Biophys. Res. Commun.*, **55**, 818 (1973).
(11) D. Yoshikami, G. Katz, E. B. Keller, and B. S. Dudock, *Biochim. Biophys. Acta*, **166**, 714 (1968).
(12) C. W. Hancher, C. F. Phares, G. D. Norelli, and A. D. Kelmers, *Biotechnol. Bioeng.*, **11**, 1055 (1969).
(13) D. W. Thomas and K. Biemann, *J. Amer. Chem. Soc.*, **87**, 5447 (1965).
(14) This aspect will be discussed elsewhere; manuscript in preparation.
(15) J. E. van Lear and L. L. Smith, *J. Org. Chem.*, **36**, 1007 (1971).
(16) M. H. Abraham. A. G. Davies, D. R. Llewellyn, and E. M. Thain, *Anal. Chim. Acta*, **17**, 499 (1957).

Contribution of Side Chain Chromophores to the Optical Activity of Proteins. Model Compound Studies. II. *p*-Hydroxyphenylglycine and *p*-Hydroxyphenylglycinamide

Julian W. Snow and Thomas M. Hooker, Jr.*

Contribution from the Department of Chemistry, University of California, Santa Barbara, California. 93106. *Received April 26, 1974*

Abstract: The optical properties and conformational stability of *p*-hydroxyphenylglycine and *p*-hydroxyphenylglycinamide have been investigated. The rotatory strengths associated with the electronic transitions of the chromophores of these molecules have been computed as a function of molecular conformation. The theoretical rotatory strengths have been used in conjunction with semiempirical conformational energy calculations and experimental optical rotatory dispersion and circular dichroism data to determine the conformation of these molecules in solution. The results of this investigation indicate that both molecules assume conformations for which the dihedral angle ψ assumes values near $-5°$ and the dihedral angle χ assumes values near 75°.

An understanding of the origin of the Cotton effects of amino acids and amino acid derivatives with side chains having chromophores with electronic transitions between 200 and 300 nm is of interest because of their possible function as a conformational probe in proteins. Model compound studies involving aromatic amino acids[1] and cyclic dipeptides[2] have been carried out previously. Experimental and theoretical spectroscopic investigations were performed on these molecules, as well as theoretical calculations of conformational potential energies, in an attempt to correlate their optical properties with conformation.

This paper reports the results of similar investigations on *p*-hydroxy-L-phenylglycine (α-amino-*p*-hydroxyphenylacetic acid) and *p*-hydroxy-L-phenylglycinamide. This amino acid was selected for study because although it is closely related to L-tyrosine, which was studied previously,[1] it is much simpler from a structural point of view. Since the aromatic ring is directly bonded to the α-carbon atom, significant stereochemical interactions should occur between the γ-hydrogen atoms of the aromatic ring and the bulky carbonyl and amino functions. Thus, the conformational freedom of these molecules should be severely restricted. Furthermore, the conformation of *p*-hydroxyphenylglycine and *p*-hydroxyphenylglycinamide can be described with one less dihedral angle than is required for the more common aromatic amino acids, so theoretical calculations are commensurately simpler. Thus, the results of these calculations should provide a good test of the theoretical techniques which are used.

Experimental Section

Procedure. The enantiomeric isomers of *p*-hydroxyphenylglycine were donated by J. H. C. Nayler. *p*-Hydroxy-D- and -L-phenylglycinamide were prepared from the amino acids by standard techniques. The amides showed only one spot when subjected to thin-layer chromatography on silica gel in acetic acid–butanol–water. Elemental analysis for carbon, hydrogen, and nitrogen showed no deviations from theoretical values greater than 0.11%. In addition, the low resolution mass spectra of both optical isomers of the amide were consistent with the products being authentic.

All samples were dried over silica gel prior to preparation of solutions. Solutions were prepared in volumetric flasks with doubly distilled water. Samples were weighed to the nearest hundredth milligram.

Absorption spectra were measured on a Cary 118C (far uv model) recording spectrophotometer. Optical rotatory dispersion (ORD) and circular dichroism (CD) spectra were measured on a Cary 60 recording spectropolarimeter equipped with a prototype Model 6003 circular dichroism accessory. Spectra were recorded at various scan rates with appropriate corresponding pen periods or time constants. The instrument slits were programmed to yield a constant spectral resolution of 15 Å. Fused silica cells with path lengths between 0.1 and 5.0 cm were used.

Optical rotation and circular dichroism data are reported as