made ambiguous by the likelihood that equilibrium effects may be at least as large as kinetic effects, unlike the case for the primary isotope substitution. This is particularly true for isotope substitution at the carbon undergoing reaction since the orbital state of the bond to the isotope is altered, and hence an equilibrium effect is to be expected.

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Preparation of a *t*-Alkyloxycarbonylhydrazide Resin and Its Application to Solid Phase Peptide Synthesis[1]

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Abstract: A new type of resin with *t*-alkyloxycarbonylhydrazide functional groups was prepared: H_2NNH-$COOC(CH_3)_2CH_2CH_2C_6H_5$ polymer. With this resin as solid support, a procedure was developed which should be useful for preparation of protected peptide hydrazides that can be purified, converted to the azides, and then condensed to other fragments to yield longer polypeptides. Combination of the conventional and solid phase approaches with retention of the best features of each is now possible. The procedure was tested by the synthesis of the crystalline tetrapeptide Z-Phe-Val-Ala-Leu-$NHNH_2$.

In solid phase peptide synthesis[3] the anchoring bond holding the peptide chain to the resin support has usually been a benzyl ester. The combination of this benzyl ester linkage with α-Boc[4] amino protection has allowed the convenient stepwise synthesis of a number of biologically active peptides by this method.[5] However, in order to release the desired peptide chain from the resin after completion of the synthesis treatment with strong acids such as HBr–TFA or anhydrous HF[6] is required and these reagents also remove most of the side chain protecting groups that are commonly used. It has, therefore, been difficult to obtain protected peptides that could be purified and then coupled to other peptide chanis by the fragment condensation method to yield larger polypeptides. The opportunity for isolation and purification of the small peptides at intermediary stages during the synthesis is an important advantage of the fragment approach. Efforts to combine the conventional and solid phase approaches, with retention of the best features of each, have been made by Anfinsen, *et al.*,[7] and by Weygand.[8] The former employed hydrazinolysis of the α-benzyl ester linkage to prepare peptide hydrazides while the latter made use of sulfhydryl-sensitive phenacyl esters to anchor the peptide to the resin. Though both of these methods are attractive, they are not without some undesirable complications and limitations and a more generally applicable method is desirable.

Recent progress in the development of acid labile urethan amino protecting groups in peptide chemistry[9] has enabled us to consider the application of *t*-alkyloxycarbonyl hydrazides as the anchoring bonds in solid phase peptide synthesis. In the following, the preparation of a new type of substituted styrene–divinylbenzene copolymer resin, $H_2NNHCOOC(CH_3)_2CH_2CH_2$–resin, is described.

Peptides can be synthesized stepwise on this type of polymer support by using the 2-(*p*-biphenyl)isopropyloxycarbonyl (Bpoc) group[10,11] for α-amino protection.

The Bpoc group can be removed at each cycle of the synthesis with very mild acid under conditions where the anchoring bond is stable. On the other hand the final peptide chain can be released as the hydrazide under more acidic conditions where side chain protecting groups remain unaffected, thus providing fragments suitable for further condensations. The feasibility of this approach was demonstrated by the synthesis of the protected tetrapeptide hydrazide, Z-Phe-Val-Ala-Leu-$NHNH_2$. The product was obtained in good yield in crystalline form with satisfactory amino acid and elementary analyses.

As indicated in Scheme I, a ketone functional group $CH_3COCH_2CH_2$– was introduced into copolystyrene–2% divinylbenzene resin beads (I) by a Friedel–Crafts reaction with methyl vinyl ketone[12] using HF as catalyst. The ketone-containing resin (II) showed an intense carbonyl absorption at 1725 cm^{-1} in the ir spectrum (see Figure 1). This functional group was then converted to a tertiary alcohol by a Grignard reaction. The absorption band at 1725 cm^{-1} disappeared completely at this stage. To prepare the *t*-alkyloxycarbonyl

(1) This work was supported in part by Grant A 1260 from the U. S. Public Health Service.
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(3) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); *Biochemistry*, 3, 1385 (1964).
(4) Abbreviations used: Boc, *t*-butyloxycarbonyl; Bpoc, 2-(*p*-biphenyl)isopropyloxycarbonyl; Z, benzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DMF, dimethylformamide.
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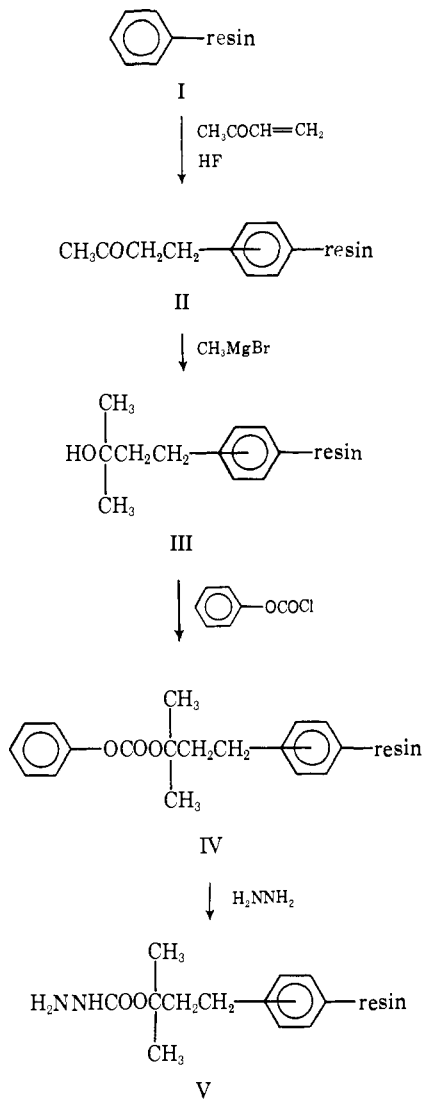
Scheme I



hydrazide resin, the alcohol resin (III) was allowed to react with phenyl chloroformate to form a phenyl carbonate resin (IV) which was then hydrazinolyzed to give *t*-alkyloxycarbonylhydrazide resin (V). Microanalysis shows that resin V contained 1.15% nitrogen while there was no detectable amount of nitrogen in resin IV. Both of these resins absorbed strongly near 1750 cm^{-1} as can be seen in Figure 1. From the nitrogen analysis, it could be calculated that the resin was substituted to the extent of 0.41 mmole of the functional group per gram of resin.

Bpoc-L-Leu[10,11] was condensed with the resin hydrazide V, using dicyclohexylcarbodiimide as coupling agent,[13] to form a protected amino acyl resin (VI) (see Scheme II). The Bpoc amino protecting group was found to be removed completely by 0.5% TFA in methylene chloride within a few minutes at room temperature while the anchoring bond was largely stable under these conditions for over 10 hr (see Figure 2). The stability of the anchoring bond under the conditions of Bpoc deprotection was such that less than a 6% loss of peptide chain would be expected even if the peptide synthetic cycle were repeated 40 times (see below).

A resin bound protected tetrapeptide hydrazide was synthesized from VI according to the general procedures

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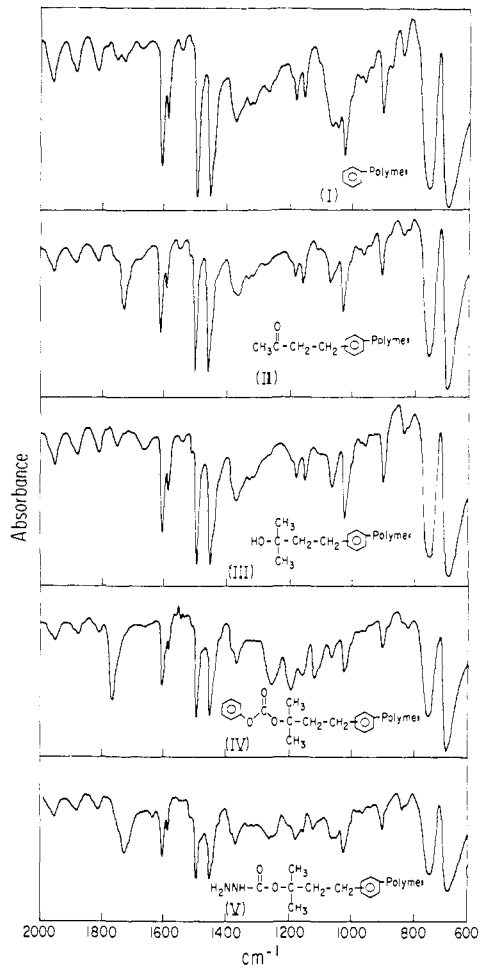

Figure 1. Infrared spectra of copolystyrene–2% divinylbenzene resin (I), 3-oxobutyl-resin (II), *t*-alkyl alcohol–resin (III), *t*-alkyl phenyl carbonate–resin (IV), and *t*-alkyloxycarbonylhydrazide–resin (V).

of solid phase peptide synthesis.[5,14] For that purpose Bpoc-L-Ala, Bpoc-L-Val, and Z-L-Phe were coupled sequentially to the growing peptide chain by the carbodiimide method. TFA (0.5%) in methylene chloride was utilized for the deprotecting agent, thus avoiding the strong acidic conditions that have previously been required. The protected peptide hydrazide (VIII) was released by treatment of the peptide resin VII with 50% TFA in methylene chloride at room temperature for 30 min. The product was obtained in pure, crystalline form in 76% over-all yield calculated from the leucine content of VI.

Experimental Section

Melting points were taken on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Infrared spectra were taken on a Perkin-Elmer infrared spectrophotometer with KBr pellets. A Gilford Model 2000 was used to measure uv absorption. Amino acid analyses were carried out on a Spinco/Beckman Model 120B amino acid analyzer according to the accelerated[15] procedure of Spackman, Stein, and Moore.[16] Elementary analyses were performed by the Microanalysis Laboratory, Rockefeller University.

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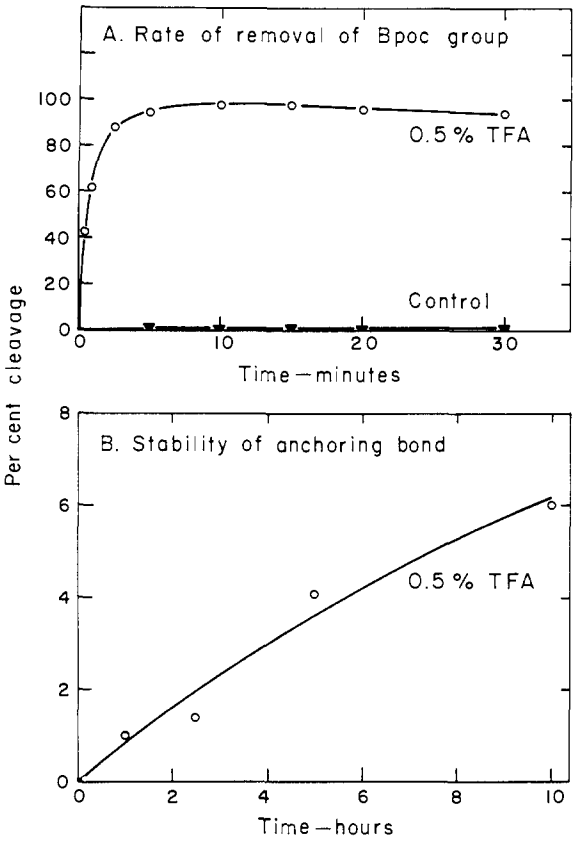


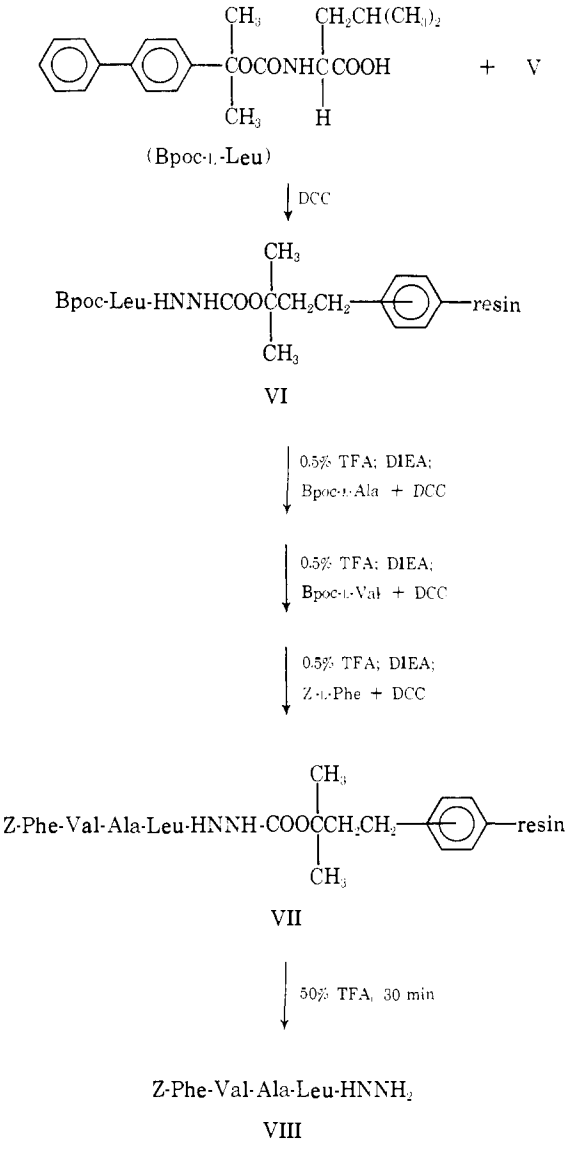
Figure 2. (A) Rate of Bpoc deprotection by 0.5% TFA in methylene chloride from Bpoc-Leu-HNNH–resin VI at room temperature. (B) The stability of the anchoring *t*-alkyloxycarbonyl bond in VI toward 0.5% TFA in methylene chloride at room temperature.

Copolymers of styrene–2% divinylbenzene (200–400 mesh) resin beads were obtained from Dow Chemical Co. Methyl vinyl ketone, TFA, and dicyclohexylcarbodiimide were purchased from Aldrich Chemical Co., Inc. Phenyl chloroformate and hydrazine hydrate were products of Distillation Products Industries. Magnesium turnings were obtained from Amend Drug and Chemical Co., Inc. Methyl bromide and anhydrous HF were supplied by Matheson Co., Inc. Bpoc-L-amino acid derivatives used were prepared in this laboratory.[11]

3-Oxobutyl–Resin (II). Styrene–2% divinylbenzene (200–400 mesh) beads were washed thoroughly with benzene, methylene chloride, 10% TFA in methylene chloride, 10% tributylamine in methylene chloride, DMF, and ethanol to remove styrene and other low molecular weight products. A 10-g sample of the washed resin (I) was then placed in a 200-ml Kel F vessel of the HF apparatus described by Sakakibara, *et al.*[17] After cooling the vessel in Dry Ice–acetone, 12.5 ml of methyl vinyl ketone was added and about 40 ml of anhydrous HF was distilled in. The mixture was stirred magnetically at room temperature for 30 min and the reaction mixture poured into 2 l. of absolute ethanol with caution. The resin particles were then collected on a glass filter and washed with ethanol, methylene chloride, benzene, DMF, methylene chloride, 3% TFA in methylene chloride, 10% tributylamine in methylene chloride, methylene chloride, dioxane, and ethanol to give 10.2 g of ketone-containing resin (II). This material absorbed strongly at 1725 cm^{-1} indicating the presence of a carbonyl function (see Figure 1). From a series of experiments, 30 min of reaction time was chosen since it gave the most desirable degree of substitution.

***t*-Alkyl Alcohol–Resin (III).** Magnesium turnings (1 g) were suspended in 200 ml of dry ether and methyl bromide was bubbled through for about 20 min until all the metal had dissolved. A suspension of ketone-containing resin (II) in benzene (10 g in 50 ml) was then added in several portions to the freshly prepared Grignard reagent. Boiling occurred each time the suspension was added. The reaction mixture was left standing at room temperature for 1 hr and then the resin was collected on a glass filter, washed with alcohol and dioxane–water, and stirred with 300 ml of an equal mixture of dioxane–1 *N* H_2SO_4 for another hour. The resin was then collected and washed with dioxane–water, dioxane, and the solvents mentioned above. The material obtained (9.5 g) showed no carbonyl absorption in the ir spectrum (see Figure 1). Microanalysis indicated that this resin had no nitrogen but had 3.84% oxygen estimated from C, H, N analyses.

Scheme II



***t*-Alkyloxycarbonylhydrazide–Resin (V).** The alcohol resin (III, 8 g) prepared above was suspended in 70 ml of methylene chloride and cooled to 0° while 5.5 ml of pyridine was added. To this suspension, 7.8 ml of phenyl chloroformate was added dropwise and then the mixture was stirred at 4° overnight. The suspension was poured onto a small amount of crushed ice and the resin particles were collected on a glass filter. After washing, a cream colored resin (IV) weighing 8.3 g was obtained. This material was hydrazinolyzed in 65 ml of DMF with 6.6 ml of hydrazine hydrate for 6 hr at room temperature. The resultant product was collected and washed to yield 8.2 g of the desired hydrazide resin (V). By microanalysis, it was shown that this resin contained 1.15% of nitrogen which corresponded to 0.41 mmole of functional group per gram of resin. The ir spectra of IV and V showed strong absorption at 1765 and 1725 cm^{-1}, respectively.

Bpoc-Aminoacylhydrazide–Resin (VI). Bpoc-L-leucine (600 mg, 1.64 mmoles) was dissolved in 8 ml of CH_2Cl_2 and was shaken with 1 g of hydrazide resin (V) for 10 min. DCC (350 mg in 8 ml of CH_2Cl_2) was then added and coupling was allowed to continue for 90 min (see Scheme II). After washing as usual, 1.18 g of Bpoc-Leu hydrazide resin (VI) was obtained which contained 0.289 mmole

(17) S. Sakakibara, Y. Shiminishi, M. Okada, Y. Kishida, *Proc. European Peptide Symposium, 8th*, Noordwijk, 1967, p 44.

of leucine per gram of resin according to amino acid analysis after hydrolysis in a mixture of dioxane–HCl.[18]

Stability of N-Bpoc Amino Protecting Group and the *t*-Alkyloxycarbonylhydrazide Anchoring Bond. A sample of Bpoc-Leu-resin (53 mg) was suspended in 1 ml of CH_2Cl_2 for a few minutes to allow the resin to swell. An equal volume of 1% TFA in the same solvent was then added and 0.1-ml samples were withdrawn at different time intervals. The resin particles were removed by filtration and washed several times with small volumes of CH_2Cl_2. The combined filtrate and washings were evaporated to dryness, dissolved in 24 ml of ethanol, and their absorbance at 254 mμ was measured to determine the amount of 2-(*p*-biphenyl)isopropyl alcohol present[10,11] with the results shown in Figure 2a. Trifluoroacetic acid was omitted from the control experiments. It can be seen that the removal of the Bpoc group was complete in about 10 min.

To see how stable the anchoring bond was under these conditions, several resin samples (VI) were prepared and suspended in 0.5% TFA in CH_2Cl_2 (30 mg/ml) in tightly capped small vials. At different times, one of the samples was taken and filtered and washed as described above. The liberated leucine hydrazide was then hydrolyzed with 6 *N* HCl at 105° for 24 hr. The leucine content of these samples is inversely related to the stability of the anchoring bond. Results of such experiments showed that there was only about 6% loss of the anchoring bond in 10 hr (see Figure 2b) which corresponded to 40 cycles of Bpoc deprotection according to the synthetic scheme described in the next section.

Z-Phe-Val-Ala-Leu-HNNH$_2$ (VIII). Bpoc-Leu-HNNH-resin (VI) (550 mg, 0.159 mmol) was placed in a peptide reaction vessel on a shaker[19] and treated as follows with 15-ml portions of solvents: (1) wash three times with CH_2Cl_2, (2) wash once with 0.5% TFA in CH_2Cl_2, (3) shake 10 min with 0.5% TFA in CH_2Cl_2, (4) wash three times with CH_2Cl_2, (5) wash once with 10% DIEA[4] in CH_2Cl_2, (6) wash three times with CH_2Cl_2, (7) wash three times with EtOH, (8) wash three times with CH_2Cl_2, (9) shake 10 min with 10% DIEA in CH_2Cl_2, (10) wash three times with CH_2Cl_2, (11) soak 10 min with 210 mg (0.64 mmole) of Bpoc-L-Ala in 6 ml of CH_2Cl_2, then add 132 mg (0.64 mmol) of DCC in 6 ml of CH_2Cl_2 and shake 90 min, (12) wash three times with CH_2Cl_2, (13) wash three times with DMF, (14) wash three times with EtOH. The cycle was repeated with 228 mg (0.64 mmol) of Bpoc-L-Val in step 11, and again with 185 mg (0.64 mmole) of Z-L-Phe. In each cycle a fourfold excess of amino acid derivative was used. The protected peptide hydrazide resin (VII) thus obtained weighed 610 mg after drying. According to the amino acid analysis, this material contained 0.232 mmole of peptide per gram of resin and had an amino acid composition of $Ala_{0.97}$ $Val_{1.00}$ $Leu_{0.97}$ $Phe_{1.05}$. The yield of the peptide at this stage was 89% calculated from the leucine content of VI.

The protected peptide hydrazide was liberated from the solid support by shaking 500 mg (0.116 mmole) of VII with 12 ml of 50% TFA in CH_2Cl_2 (v/v) at room temperature for 30 min. The resin was removed by filtration and washed with a few milliliters of CH_2Cl_2. After removal of the solvent by evaporation under reduced pressure, the peptide hydrazide was obtained as a white powder. It was then crystallized from methanol by addition of ether to give 60 mg (0.101 mmol) of the product melting at 255–257°. It had an amino acid composition of $Ala_{0.99}$ $Val_{1.00}$ $Leu_{1.04}$-$Phe_{1.00}$.

Anal. Calcd for $C_{31}H_{44}N_6O_6$ (596.71): C, 62.30; H, 7.43; N, 14.08. Found: C, 62.21; H, 7.40; N, 14.10.

Acknowledgments. We wish to thank Miss D. M. Cohen for her technical assistance in the preparation of Bpoc-amino acid derivatives, Miss U. Birkenmaier for carrying out the amino acid analyses, and Mr. S. T. Bella for performing the microanalysis.

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The Pepsin-Catalyzed Hydrolysis of Bis-*p*-nitrophenyl Sulfite and Its Inhibition by Diphenyl Sulfite at pH 2

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Abstract: Bis-*p*-nitrophenyl sulfite, BNPS, is an excellent substrate for the proteolytic enzyme pepsin at pH 2. The enzyme-catalyzed hydrolysis of this sulfite proceeds approximately 10^3 times faster than that of other known sulfite esters. This rate acceleration is apparently due principally to the high value of k_{cat}, the catalytic rate constant, for the enzymatic hydrolysis of BNPS. The Michaelis constant for the pepsin-catalyzed hydrolysis of the nitro-substituted aromatic sulfite ester is not very different from that which is observed in the case of the unsubstituted ester diphenyl sulfite, DPS. We have studied the inhibition of the enzymatic hydrolysis of BNPS by added DPS. The inhibition constant, K_I, which we have measured in this way for DPS differs appreciably from the Michaelis constant we have found for the pepsin-catalyzed hydrolysis of DPS.

Recently, much evidence has been presented that the Michaelis constants, K_M, obtained from kinetic analyses of pepsin-catalyzed reactions are simple dissociation constants.[3–5] This would imply that in the reaction scheme represented by eq 1 which is likely to apply for pepsin-catalyzed hydrolyses,[5–8] k_2 is the rate-

(1) Predoctoral Fellow of the National Science Foundation.

(2) Fellow of the Alfred P. Sloan Foundation to whom inquiries concerning this article should be addressed.

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(8) The abbreviations used in this paper are: acetyl, Ac; diphenyl sulfite, DPS; bis-*p*-nitrophenyl sulfite, BNPS; dibromotyrosine, Di-BrTyr; phenylalanine, Phe. v_0 is the initial rate of an enzymatic reaction, and k_{cat} and K_M are the catalytic rate constant and Michaelis constant, respectively, for such a reaction. K_I^c is the inhibition constant calculated for acetonitrile if this substance is treated as a competitive inhibitor. All amino acid residues are of the L configuration unless otherwise specified. ES represents a Michaelis complex and ES′ is a covalent species.