*IDENTIFICATION OF HISTIDINE 64 IN THE ACTIVE SITE OF SUBTILISIN**

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Although the amino acid sequences of two subtilisins[1] do not resemble those of the pancreatic proteinases chymotrypsin[2] and trypsin,[3, 4] the two groups of enzymes are similar in that they are both inhibited by reaction with diisopropylfluorophosphate at a single specific serine residue.[5] Further, a specific histidine residue has been identified in the active sites of trypsin and chymotrypsin by reaction with the specific alkylating agents TLCK[6, 7] and TPCK.[8] Although these reagents do not inhibit the subtilisins,[9] kinetic data do suggest involvement of an un-ionized histidine residue in the active sites of these enzymes.[10]

Shaw and Ruscica[11] have found that the increased reactivity of the bromomethyl ketone of carbobenzoxy-L-phenylalanine (ZPBK) is sufficient to inhibit the subtilisins. There is close agreement between the loss of activity, incorporation of tritium from ^{3}H-ZPBK, and loss of histidine with subtilisin BPN′. The reagent is stereospecific with only the L-form being reactive with the enzyme. That the reactive histidine must be involved in the enzymic activity is demonstrated by the finding that subtilisin BPN′ treated with ZPBK no longer reacts with diisopropylfluorophosphate. After performic acid oxidation, 3-carboxymethylhistidine was identified. We wish to report on the identification of the active-site histidine residue in the subtilisins with the use of ZPBK.

Materials and Methods.—Chemicals: Subtilisin Carlsberg was obtained from Novo Pharmaceutical Company, Copenhagen, Denmark (batch 60420), and subtilisin BPN′ (as Nagarse) from Biddle Sawyer Corp., New York. ZPBK and ^{3}H-ZPBK were synthesized by the method of Shaw and Ruscica.[11]

Radioactivity measurements: Radioactivity of the tritium-labeled protein or peptides was measured in a Packard (model 3003) liquid scintillation spectrometer. Liquid samples of 20–100 μl (usually 50 μl) were counted in 10.0 ml of Bray's solution,[12] with corrections for background but not for differences in efficiencies with the different solvents or different sized aliquots. To locate radioactive peptides after preparative chromatography or electrophoresis, guide strips that had been sprayed with ninhydrin or the Pauly reagent[13] were cut into small pieces and counted as described above in 10.0 ml of a scintillation solvent that contained 6 gm of PPO and 0.3 gm of dimethyl POPOP in 1 liter of toluene. In later stages of the work, a Beckman solubilizer BBS-3 was added to the toluene scintillation solvent[14] to improve the counting efficiency.

Peptide separation: Peptides were separated either by gel filtration on Sephadex G-75 or G-25 in formic acid–propanol–water (30:20:50 by volume) and detected with ninhydrin after alkaline hydrolysis.[15] Paper chromatography in n-butanol–acetic acid–water (200:30:75 by volume) (BAW) and paper electrophoresis at pH 1.9 were used preparatively. Peptides were eluted from paper with 35% acetic acid and, unless otherwise specified, were dried on a rotary evaporator. Peptide maps were run with electrophoresis at pH 1.9 and chromatography in BAW.

Amino acid analysis: Samples were hydrolyzed at 110° for 24 hr in evacuated glass tubes with 6 *N* HCl containing a drop of 5% phenol in water. Analyses were performed with the Spinco automatic amino acid analyzer model 120 B with an accelerated system.[16, 17]

Results.—Reaction of ^{3}H-ZPBK with subtilisin Carlsberg: Most of the work

was performed with subtilisin Carlsberg. About 4.0 ml of enzyme (100 mg per ml) was dialyzed against 1 liter of 0.02 *N* sodium acetate containing 0.01 *M* $CaCl_2$, pH 6.0, for 18 hours. The dialysis medium was changed after six hours. The protein (11.8 μmoles) was then added to 219.0 ml of 0.1 *M* Tris-chloride containing 0.02 *M* $CaCl_2$, pH 7.0, and 84.4 ml of dioxane. The mixture was allowed to react at 37° with 536 μmoles of ^{3}H-ZPBK (28.7×10^6 cpm) in 15.6 ml of dioxane for 25.5 hours. The initial pH of 6.8 dropped to 6.4 at completion of the reaction. A control (at 1% of the quantity) was kept under identical conditions, but without H-ZPBK. Enzyme was assayed in duplicate with ATEE.[10]

A representative experiment of the course of the reaction of ^{3}H-ZPBK with subtilisin is shown in Figure 1. In this experiment, 91 per cent of the enzyme activity was lost in 21 hours, whereas in the large-scale experiment described above, there was a loss of 87 per cent of the enzyme activity in 25.5 hours.

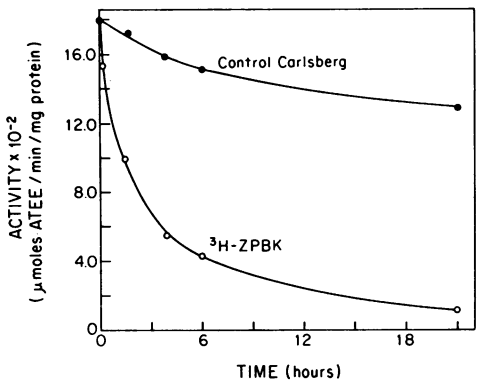

FIG. 1.—Time courses of the reaction of ^{3}H-ZPBK with subtilisin Carlsberg under the conditions described in the text.

Upon completion of the reaction, unreacted ^{3}H-ZPBK was extracted twice with 95 ml ether and 6 *N* HCl added to pH 2.5. The mixture was dialyzed for 44 hours against 10^{-3} *M* HCl and freeze-dried. The yield of derivatized protein was 105 mg. Analysis after hydrolysis gave the following results (values from the sequence are given parenthetically): Lys 8.6(9); His 4.3(5); Arg 4.0(4); Asp 29.2(28); Thr 19.6(19); Ser 33.1(32); Glu 12.1(12); Pro 10.0(9); Gly 34.9(35); Ala 41.1(41); Val 29.8(31); Met 5.0(5); Ile 9.1(10); Leu 16.0(16); Tyr 13.1(13); Phe 4.0(4). Analysis after performic acid oxidation gave a value of 0.3 (1) for 3-carboxymethylHis. Residues were calculated on the basis of 4 Arg and 16 Leu; Trp was not determined. The low values for Val and Ile are due to incomplete hydrolysis.[18] The results indicate a loss of 0.7 residue of histidine, with all other residues the same within experimental error. The radioactivity showed incorporation of 0.77 mole of ^{3}H-ZPBK per mole of protein (Table 1), which is in accord with the loss of histidine and enzymic activity.

Tryptic hydrolysis: The five histidine residues of subtilisin Carlsberg were obtained in three tryptic peptides of varying size:[18] peptide T-5 contained residues 39, 64, and 67; peptide T-10, residue 226; and peptide T-11, residue 238.[19]

For tryptic digestion, the labeled protein was dissolved in 18 ml of 35 per cent formic acid and precipitated with an equal volume of 20 per cent TCA. After one hour, the precipitate was worked up and digested with TPCK-treated trypsin,[20] as previously described,[21] by using 150 μl of a 1 per cent solution of trypsin added initially and at 32 minutes. After 62 minutes, formic acid was added.

The digest was applied to a 3.8 × 150-cm column of Sephadex G-75 at room temperature and fractions of 3.3 ml were collected at a flow rate of 30 ml per

TABLE 1. *Isolation of radioactive peptides.*

Step	Fraction	μMoles radioactive protein or peptide	^{3}H		Moles ^{3}H-ZPBK per mole protein or peptide
			Total cpm	μMoles	
1	Starting material	11.8	28.7×10^6	536	45
2	Dialyzed ^{3}H-ZPBK subtilisin Carlsberg before TCA denaturation	3.81	1.58×10^5	2.95	0.77
3	^{3}H-ZPBK subtilisin Carlsberg after tryptic digestion	——	4.5×10^4	0.85	——
4	Tryptic peptides T-5 plus T-10	~1.65	3.18×10^4	0.59	0.36
5	Tryptic peptide T-5 after BrCN cleavage	1.25	2.20×10^4	0.41	0.33
6	Peptide T-5-C-1	0.48	1.61×10^4	0.30	0.63
7	Peptide T-5-C-1-P-1	0.13	3.79×10^3	0.07	0.54
8	Peptide T-5-C-1-P-1-Cp-1	0.009	2.49×10^2	0.005	0.56

hour. Aliquots of 250 μl from alternate fractions were analyzed for peptides. Radioactivity was measured on 100-μl aliquots generally from every fifth fraction and from alternate fractions in the peak areas.

As shown in Figure 2, there were several ninhydrin peaks, but the bulk of the radioactivity was present in fraction A, which, as indicated by peptide maps, contained the 66-residue peptide T-5 and the 51-residue peptide T-10.[19] Fraction B (Fig. 2) contained some radioactivity and the same peptides, although more of T-10 and less of T-5. Inasmuch as peptide T-10 contains two residues of methionine, and peptide T-5 none, cyanogen bromide cleavage should split T-10 into fragments that could be readily separated from T-5 by gel filtration.

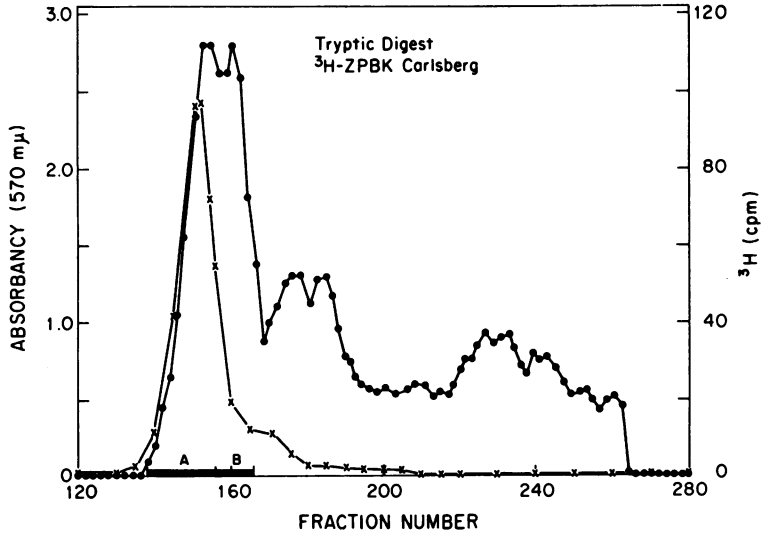

FIG. 2.—Elution profile of peptides from tryptic digest of ^{3}H-ZPBK subtilisin Carlsberg after gel filtration on Sephadex G-75, as described in the text. Fractions A and B were pooled as indicated by the solid bars on the abscissa.

(●) Ninhydrin color; (×) tritium cpm per 100 μl (corrected for background).

Fraction A plus B was dried and dissolved in 2.0 ml of 70 per cent formic acid. Analysis indicated a total of 3.8 μmoles of methionine. A 100-fold molar excess of BrCN in 88 per cent formic acid was added and the mixture was kept in the dark for 22 hours at room temperature. Ten volumes of water were added and the mixture was dried. The residue was dissolved in 2 ml of 40 per cent formic acid and resolved on a 3.8 × 150-cm column of Sephadex G-75. Fractions of 3.8 ml were collected at a flow rate of 35 ml per hour.

As shown in Figure 3, only the earliest peptide fraction contained radioactivity, which indicates that peptide T-5 (66 residues) contained the labeled histidine residue. Analysis confirmed the identification as peptide T-5 (Table 2).

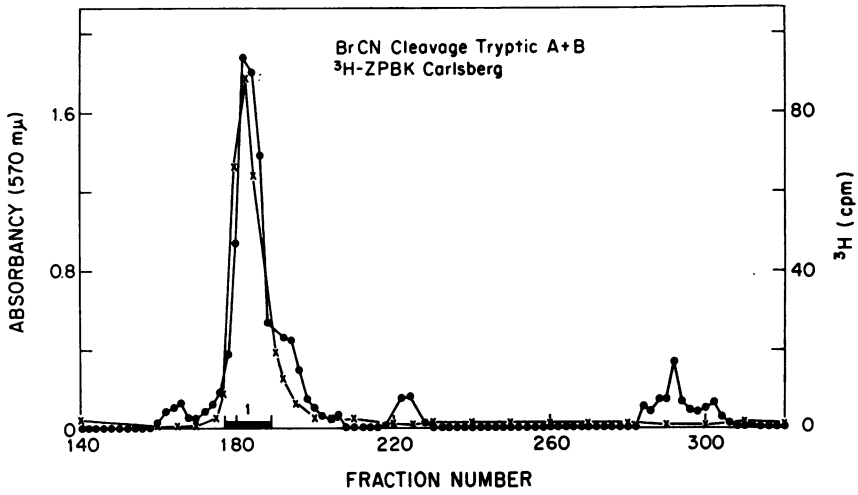

FIG. 3.—Elution profile on Sephadex G-75 of peptides from pooled fractions A and B (Fig. 2) after cleavage with BrCN. The fractions were pooled as indicated by the solid bar on the abscissa.

(●) Ninhydrin color; (×) tritium cpm per 100 μl (corrected for background).

As shown in Table 1, there was a significant loss in the radioactivity recovered after TCA-denaturation of the derivatized protein (approximately 29% recovery). This could be explained in part by about a 55 per cent loss in protein as judged by the low recovery of peptide T-5 (1.65 μmoles of the initial 3.81 μmoles). However, the apparent low level of radioactivity in steps 4 and 5 (Table 1) may be due to loss of counting efficiency. In any event, the level was restored to that expected at step 6 (Table 1), and the loss of histidine as judged by analysis (Table 2) of peptide T-5 was 0.95 residue. Furthermore, during each cleavage step (Table 1, steps 4, 5, and 6), all the radioactivity was associated with only one ninhydrin peak, and the recovery was about 70 per cent at each step, which is satisfactory considering losses in handling and sampling.

Chymotryptic and papain hydrolysis: The pooled fractions containing the radioactive peak, shown in Figure 3, were dried and then dissolved in 1.0 ml of 30 per cent formic acid. Ammonia was added to pH 8.5, which produced a finely dispersed precipitate that persisted for several hours during the chymotryptic digestion. To approximately 1.2 μmoles of peptide, about 1 mg of α-

TABLE 2. *Amino acid analysis of radioactive peptides.**

Amino acid	Peptide T-5, residues 28–94	Peptide T-5-C-1, residues 58–70	Peptide T-5-C-1-P-1, residues 58–67	Peptide T-5-C-1-P-1-Cp-1, residues 58–65
Lys	1.0(1)	0.1	0.3	0.3
His	2.1(2)	1.0(1)	1.1(1)	0.1
^{3}H-ZPBK His	0.33(1)†	0.63(1)†	0.54(1)†	0.56(1)†
Asp	7.3(8)	3.1(3)	2.8(3)	2.3(3)
Thr‡	6.3(6)	2.2(2)	1.8(2)	1.0(1)
Ser‡	5.9(4)	0.7	0.5	0.9
Glu	2.3(2)	0.4	0.3	0.7
Pro	2.3(2)	0.4	0.2	
Gly	10.0(10)	3.9(4)	3.1(3)	3.1(3)
Ala	10.9(10)	1.2(1)	0.4	
Val	9.4(11)§	1.1(1)	0.2	0.3
Met	0.6(0)	0.2	0.1	
Ile	1.3(1)	0.2	0.1	0.1
Leu	5.1(5)	0.5	0.2	
Tyr	2.4(2)	0.1		
Phe	1.0(1)			
Total no. of residues	66	13	10	8

* An average value of the stable amino acids was used to determine the one-residue level. Theoretical values[1] from the sequence are given parenthetically.

† From the amount of tritium present.

‡ Thr and Ser corrected for 5% and 10% destruction, respectively.

§ Value is low due to incomplete hydrolysis of a Val-Val bond.

chymotrypsin (Worthington, 3 times crystallized) in 100 μl 10^{-3} *M* HCl was added initially and after seven hours when the solution was almost completely clear. After 23 hours at 40°, the reaction was terminated by adding formic acid to pH 2.5. The dried chymotryptic digest of peptide T-5 was fractionated on a 1.8 × 150-cm column of Sephadex G-25. Fractions of 2.0 ml were collected at a flow rate of 22 ml per hour.

As shown in Figure 4, there was only one radioactive peak containing essentially all of the counts. A peptide map of this material indicated only one peptide; it gave a positive reaction with the Pauly reagent, but was ninhydrin-negative. Its composition (Table 2, peptide T-5-C-1) indicated 13 residues extending from residue 58 through 70; however, there was the expected loss of one of the three histidine residues. The sequence of this peptide is as follows: Asn-Thr-Asp-Gly-Asn-Gly-His-Gly-Thr-His-Val-Ala-Gly. The level of radioactivity present (Table 1, step 6) indicated 0.63 mole of tritium per mole. The composition of peptide T-5-C-1 indicates scission between Gly-Thr, an unusual bond for chymotryptic hydrolysis; however, the digestion was exhaustive since 20 per cent by weight of α-chymotrypsin was present at 40° for 23 hours. Although chymotryptic digestion did remove histidine residue 39, the radioactive peptide still contained histidine residues 64 and 67.

Previous work[22] indicated that papain cleaved between the two histidine residues in a similar peptide (residues 58 through 67) obtained from the chymotroyptic digest of subtilisin Carlsberg. Therefore, the dried peptide T-5-C-1 was dissolved in 1.0 ml of 0.1 *M* KCN at pH 6 and treated with 1 mg of papain for 20 hours, the reaction being terminated by addition of acetic acid to pH 3. Mapping indicated the appearance of several new peptides that were separated by paper chromatography for 20 hours.

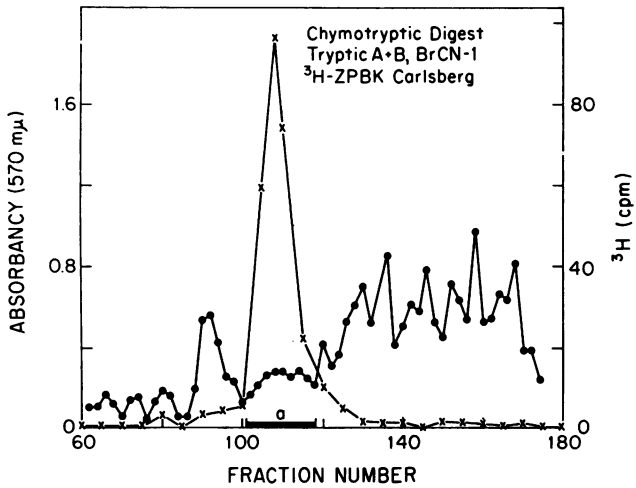

FIG. 4.—Elution profile of peptides on Sephadex G-25 from the chymotryptic digest of radioactive peptide T-5. Several additional ninhydrin peaks, emerging later, are omitted since no radioactivity was present. The fractions were pooled as indicated by the solid bar on the abscissa.
(●) Ninhydrin color; (×) tritium cpm per 100 μl (corrected for background).

From 0.4 μmole of the radioactive chymotryptic peptide T-5-C-1, we obtained, as the major radioactive product, a peptide lacking the three carboxyl-terminal residues (Table 2, peptide T-5-C-1-P-1). Thus, this peptide comprised residues 58 through 67 (Asn-Thr-Asp-Gly-Asn-Gly-His-Gly-Thr-His) and contained essentially the same level of radioactivity as the previous peptides (Table 1). There was a loss of 0.9 residue of histidine (Table 2).

About 0.04 μmole of the peptide was digested for 24 hours at 40° in 0.06 *M* NH_4HCO_3 with 150 μg of carboxypeptidase A (Worthington COADFP). Separation of the products by preparative electrophoresis yielded free histidine and threonine (identified by comparison with the authentic amino acids) and only trace amounts of glycine, alanine, and serine; none of these amino acids contained radioactivity.

The residual peptide, which was radioactive, was eluted and analyzed after acid hydrolysis. The composition (Table 2) indicated that this peptide, T-5-C-1-P-1-Cp-1, contained residues 58–65, with histidine itself completely lacking. The level of contaminating amino acids eluted from paper was high; however, the results are conclusive since serine and glutamic acid, the two major contaminants, were not present in peptide T-5-C-1-P-1 (Table 2). Since the level of radioactivity in this peptide was approximately the same as in the earlier peptides (Table 1), it is evident that histidine 64, the only histidine residue remaining, was labeled with the radioactive reagent.

Subtilisin BPN': This enzyme, which had been 65 per cent inhibited with ^{3}H-ZPBK as described above, was cleaved with cyanogen bromide. Analysis indicated 93 per cent destruction of methionine. The BrCN fragments were fractionated on a 1 × 200-cm column of Sephadex G-100 in 30 per cent acetic acid. The main radioactive peak was dried and digested with trypsin as pre-

viously described.[23] The digest was fractionated on a 1.8 × 200-cm column of Sephadex G-50 in 30 per cent acetic acid and yielded a single radioactive peak. From the behavior on Sephadex and amino acid analysis, it was evident that the labeled peptide corresponded to the previously designated peptide BrCN-2-T-1.[23] This peptide was purified by paper chromatography in solvent II.[24] Analysis after performic acid oxidation[25] and hydrolysis was in accord with the expected composition (in parentheses) of residues 51 through 94: Lys 0.9(1); His 1.3(2); 3-carboxymethylHis 0.2; Arg 0.1(0); Asp 6.0(6); Thr 2.7(3); Ser 4.9(5); Glu 2.2(2); Pro 2.6(3); Gly 5.0(4); Ala 6.1(6); Val 5.6(6); Ile 1.2(1); Leu 2.8(3); Tyr 0.7(1); Phe 0.9(1); radioactivity 0.62(0.65). Tyr was determined on an unoxidized sample.

From these results, it can be seen that one histidine residue was converted to the 3-carboxymethyl derivative, and this was demonstrated to be either residue 64 or 67. From the homology of the two subtilisins, the active-site histidine is presumed to be residue 64.

Discussion.—From the above results, it is evident that carbobenzoxy-L-phenylalanine bromomethyl ketone is an effective, covalently bound inhibitor of both subtilisins. In all cases, we have found good stoichiometry among the degree of inhibition, the incorporation of tritium, and the loss of histidine after inhibition by ^{3}H-ZPBK. The histidine residue becomes substituted at the 3-position of the imidazole ring, since mild performic acid oxidation followed by acid hydrolysis yields 3-carboxymethylhistidine. Although the recovery has not been quantitative (20–30% recovery), the same derivative has been identified in both subtilisins. It is noteworthy that for trypsin and chymotrypsin, it is also the 3-position that is substituted by TLCK[7] and by TPCK and other reagents.[26]

The residue at the active site in subtilisin Carlsberg has been demonstrated by the isolation of the octapeptide comprising residues 58 through 65 which contains the labeled histidine of residue 64. From subtilisin BPN′, a labeled peptide was isolated that contained all the radioactivity. This peptide contains the two histidine residues, 64 and 67, which implies that histidine 64 is also involved in the active site in view of the homology in sequence of the two subtilisins.

In earlier work, it was demonstrated that the reactive serine is at residue 221 and in a constant sequence from residues 218 through 240.[1, 23] In contrast, residue 63, next to the active histidine, is glycine in subtilisin Carlsberg[1] and serine in subtilisin BPN′.[23] Furthermore, although the sequence in both enzymes is constant from residues 64 through 75, there are 10 differences from residues 50 through 63, including a deletion in subtilisin Carlsberg. (To preserve the homology, we used the numbering system for the BPN′ enzyme.[1]) Inasmuch as the two enzymes differ in absolute activity,[10] and to a minor extent in substrate specificity,[27] it is tempting to infer that one or more of the differences in sequence at the NH_2-terminal side of histidine 64 may be responsible.

It is obvious that histidine 64 is far removed from the reactive serine 221 in the linear sequence of the polypeptide chain. This is strikingly similar to the situation in α-chymotrypsin and trypsin, where the reactive histidine, residues 57 and 46, respectively, is also close to the amino terminal and far removed from the reactive serine, residues 195 and 184, respectively.[28]

Summary.—Reaction of the tritium-labeled bromomethyl ketone of carbobenzoxy-L-phenylalanine has been used to identify the reactive histidine in the subtilisins. After suitable degradation of the labeled proteins by proteolytic hydrolysis and cyanogen bromide cleavage, peptides were isolated that proved to contain histidine residue 64 substituted in the 3-position.

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[6] The following abbreviations are used: TLCK, L-1-chloro-3-tosylamido-7-amino-2-heptanone; TPCK, L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone; ZPBK, carbobenzoxy-L-phenylalanine bromomethyl ketone; PPO, 2,5-diphenyloxazole; dimethyl POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene; TCA, trichloroacetic acid; BrCN, cyanogen bromide; ATEE, N-acetyl-L-tyrosine ethyl ester; BAW, n-butanol–acetic acid–water; Tris-chloride, tris(hydroxymethyl)aminomethane-chloride; KCN, potassium cyanide.

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