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Selenotyrosine and Related Phenylalanine Derivatives

Howard E. Ganther*

Department of Nutritional Sciences, 1415 Linden Drive, University of WI Madison, Madison, WI 53706, USA

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Abstract—A new series of Se-substituted phenylalanine derivatives has been synthesized having the para position of the phenyl ring substituted by selenocyanate (-SeCN), seleninic acid (-SeO_2H), or selenol (-SeH) functional groups. The starting material for synthesis was 4′-aminophenylalanine, which is readily available in DL- or L- forms. Selenium was incorporated into the ring by reacting the unprotected amino acid with nitrous acid, followed by reaction of the diazotized aromatic amine with potassium selenocyanate at pH 4–5 to give phenylalanine selenocyanate. The selenocyanate derivative was converted to the selenol directly by reduction with sodium borohydride, or oxidized to the seleninic acid, which was then reduced to the selenol. Alkylation of the selenol ('selenotyrosine') gave the selenoether derivatives of phenylalanine [(Phe-SeR), R = methyl or allyl], and air oxidation of the selenol gave the diselenide. Mild oxidation of the selenoether 4′-(MeSe)Phe with peroxide gave the selenoxide derivative, 4′-[Se(O)Me]. Because of their stability and useful redox properties, aromatic selenoamino acids can be used as synthetic analogues to increase chemical functionality in proteins or peptides, and have potential pharmaceutical or nutritional applications. The possibility that aromatic selenoamino acids could be formed metabolically through reactions of reactive selenium intermediates with aromatic amino acid residues is discussed.

Introduction

The nutritional roles of selenium (Se) at sub-ppm dietary levels are well established.[1] Cancer chemopreventive activity demonstrated with supranutritional levels of Se in animals[2] and Se supplements in humans[3] has drawn much attention. The increasing use of long-term Se supplementation by large numbers of persons increases the need for a fuller understanding of the chemistry of Se relevant to its biological activities. Selenium compounds undergo extensive metabolism and form highly reactive intermediates. Generation of hydrogen selenide and increased formation of methylated selenide derivatives is associated with supranutritional levels of Se intake.[4] Minute quantities of hydrogen selenide also provide Se needed for specific co-translational synthesis of selenoproteins containing selenocysteine; the functions of Se as an essential trace element at normal dietary levels are known to involve at least a dozen selenoproteins.[5] Translational incorporation of selenomethionine in place of methionine occurs non-specifically, reflecting the relative abundance of these amino acids. No other selenoamino acids are known to occur in proteins, but Se-methylselenocysteine and some other analogues of non-protein sulfur amino acids are found in plants and selenized garlic.[6] Se-methylselenocysteine and Se-allylselenocysteine are more active than selenomethionine in cancer chemoprevention studies.[7]

Aromatic selenoamino acids offer interesting possibilities for study, but very few have been synthesized and none are known to occur naturally. In general, aromatic Se compounds are more stable than aliphatic Se compounds.[8] Aromatic selenoamino acids would be useful synthetic analogues for studies of protein or peptide chemistry, and possibly could have pharmaceutical or nutraceutical applications. Thiotyrosine, an analogue of tyrosine having the phenolic oxygen replaced by sulfur, was synthesized almost a century ago.[9] The present article describes the synthesis and some properties of selenotyrosine and related phenylalanine derivatives. This group of aromatic selenoamino acids was chosen for their chemical simplicity and stability. The corresponding sulfur derivatives of phenylalanine already are known.[10,11] The starting material for synthesis was 4′-aminophenylalanine, which is readily available in DL- or L- forms from commercial sources or by synthesis.[12] Upon reaction with nitrous acid, selective diazotization of the *p*-amino group on the aromatic ring is possible, so that the α-amino group of the amino acid is unaffected.[13] The reaction of potassium selenocyanate with a diazotized aromatic amine is a well-established method

*Tel.: +1-608-262-2727; fax: +1-608-262-5860; e-mail: hganther@facstaff.wisc.edu

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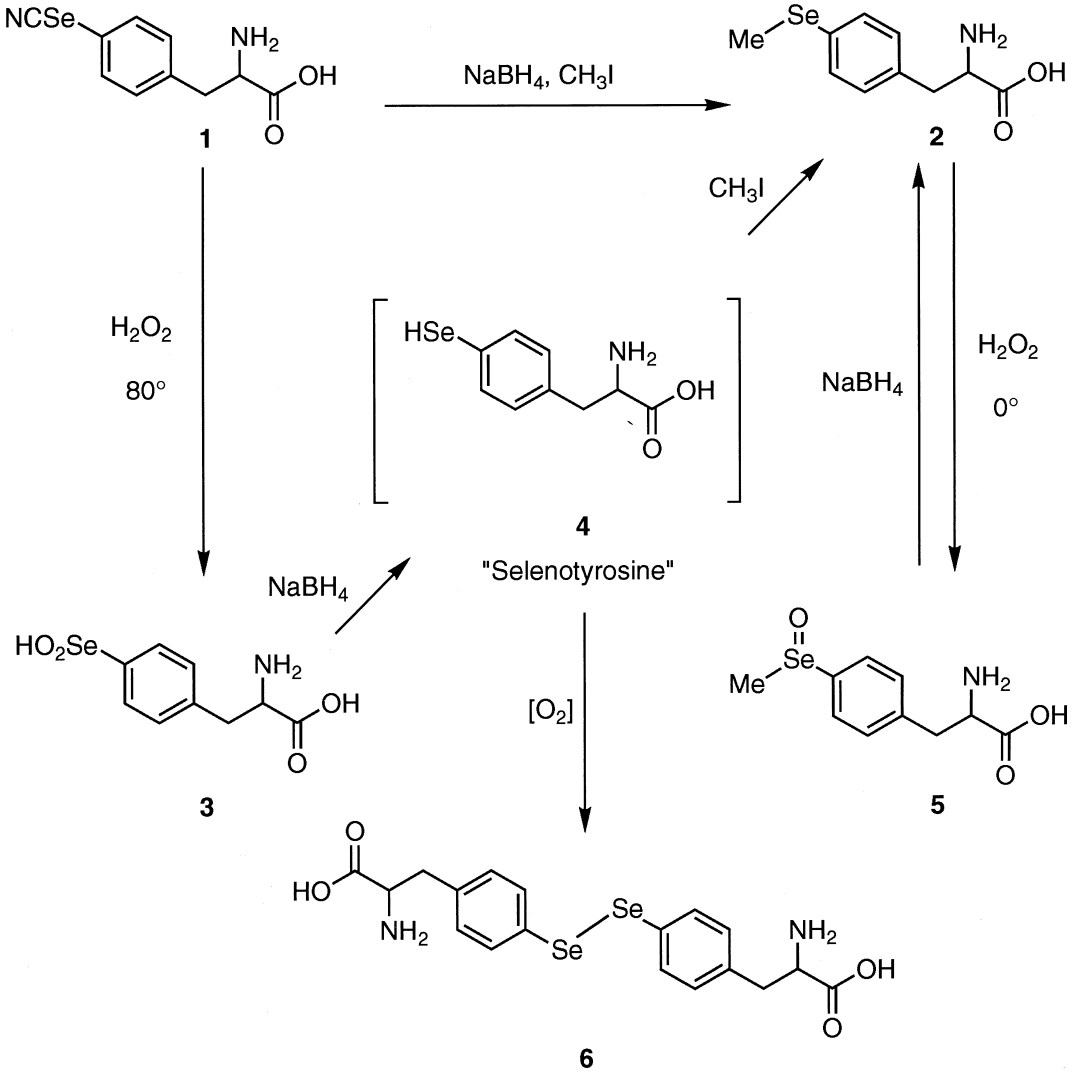
for attaching Se to an aromatic ring.[8] By diazotization of the *p*-amino group of 4′-aminophenylalanine and reaction with potassium selenocyanate, the selenocyanate derivative of phenylalanine was obtained, from which selenotyrosine and other derivatives were synthesized (Scheme 1).

Results

Selenenylation of phenylalanine ring

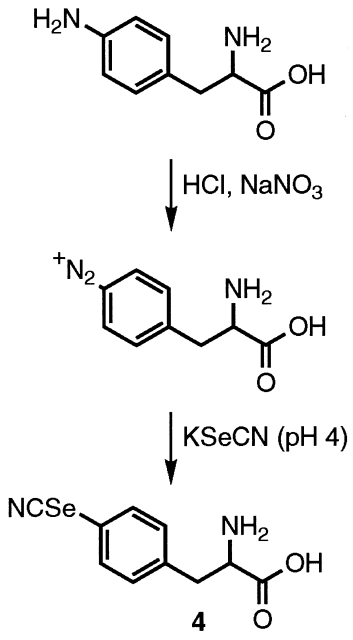
The 4′-amino group of 4′-aminophenylalanine was diazotized using the optimal conditions described by Gram et al.,[13] then reacted with KSeCN (Scheme 2). The selenylation reaction uses KSeCN, an inexpensive Se reagent; it is readily prepared from elemental Se,[8] thus KSeCN can be labeled with ^{75}Se or other isotopes of Se for synthesis of isotopically labeled selenoamino acid derivatives if desired. Under the strongly acidic conditions needed for diazotization, KSeCN is unstable and releases elemental Se; for this reason the selenocyanylation reaction was carried out at pH 4. Optimization of reaction conditions such as reagent concentrations and pH to increase yield might be worthwhile in the case of isotope labeling reactions, but was not explored. The purification method employed a polymeric XAD-4 column under conditions that retain aromatic aminoacids.[14] The strategy was to isolate the selenocyanate intermediate in high purity (at the expense of yield) using conditions as mild as possible, thus minimizing side reactions that might complicate subsequent reactions. The filtered reaction mixture was applied to a large XAD column to adsorb the aromatic selenocyanate. After washing with water and 20% methanol, elution with 40% methanol gave a ninhydrin-positive product having an R_f higher than the parent 4′-aminophenylalanine and similar to phenylalanine. The UV spectrum (Fig. 1) showed features similar to phenylselenocyanate and phenylthiocyanate.[15] HPLC analysis showed a major peak of 4′-(SeCN)Phe **1** (identified by mass spectroscopy) plus small amounts of more polar impurities. A more polar ninhydrin-positive impurity observed by TLC of the 40% methanol eluate showed a Se peak at $m/z = 434.0$ corresponding to $[M+H]^+$ [^{80}Se] for



Scheme 1.

$C_{19}H_{19}N_3O_4Se$, as well as a Se-containing peak at $m/z = 217.5$ for the doubly-charged molecule. These properties are consistent with a biphenyl derivative that would be formed by further coupling of diazotized aminophenylalanine with 4′-(SeCN)Phe, the initial product in the diazotization reaction.

In order to remove dimeric or polymeric by-products (expected to be more cationic), the XAD eluate was passed through a Dowex 50 (NH_4^+) cation exchange column (the sample was first adjusted to neutral pH so that the amino acid would be in its isoelectric form to minimize retention on the column). A brown band of material remained at the top of the column and the nearly colorless eluate of pure 4′-(SeCN)Phe **1** was collected. This work up procedure gave good purification but left considerable amounts of 4′-(SeCN)Phe on the column, as shown by subsequent analysis. Alternative procedures such as Fluorosil column chromatography gave good purification but resulted in deposition of a glassy solid on the walls of the glass fraction collection tubes. Polymerization of organic selenocyanates may be catalyzed by trace metals present in Fluorosil. Dilute aqueous methanol solutions of the purified selenocyanate were stable when stored in a refrigerator, and this stock solution of **1** was used for the synthesis of other derivatives.



Scheme 2.

Synthesis of DL-4′-methylselanylphenylalanine: [4′-(MeSe)Phe] (2)

The reduction/methylation of selenocyanate **1** was carried out in one step under nitrogen (Scheme 1, top), using sodium borohydride[16] and excess methyl iodide. The pH was maintained between 6 and 7 so that the intermediate selenol was in the unprotonated (selenolate) form and the amino group remained protonated. TLC analysis of the reaction mixture showed a single ninhydrin-positive spot ($R_f = 0.77$) having a higher mobility than that of the parent compound ($R_f = 0.56$) or a phenylalanine standard ($R_f = 0.64$). HPLC analysis with diode array detection showed a major peak (retention time 8.3 min) having a distinctive UV spectrum with maxima at 251 and 266 nm (Fig. 2). This peak was identified as 4′-(MeSe)Phe **2** on the basis of mass spectroscopy and other methods. Alternatively, selenoether **2** was prepared conveniently in two successive reactions from phenylalanine seleninic acid **3** (see below). After reduction with sodium borohydride to give the

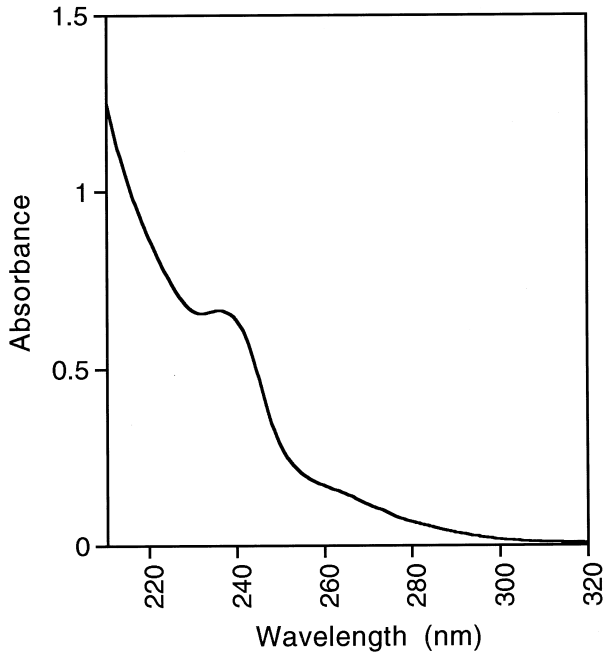


Figure 1. Absorption spectrum of 4′-(selenocyanato)phenylalanine **1** (0.0928 mM in 65% methanol).

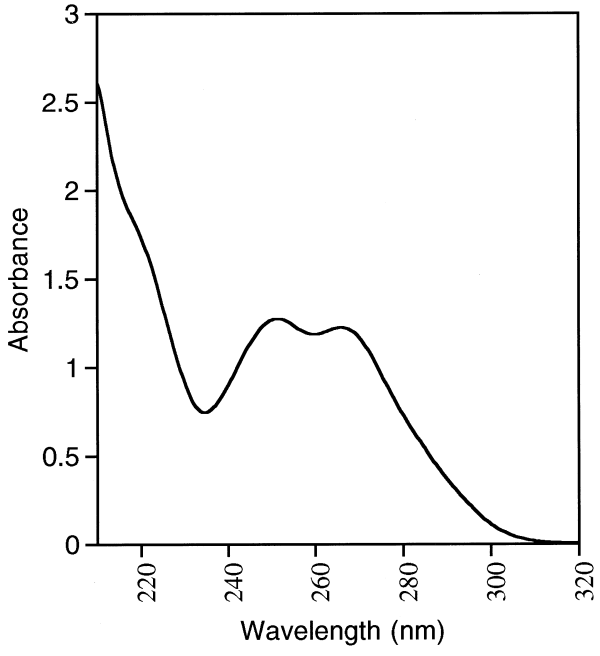


Figure 2. Absorption spectrum of 4′-(methylselenanyl)phenylalanine **2**. The crystalline compound was dissolved in 65% methanol and scanned against a solvent reference solution. The sample concentration was 0.167 mM, determined by Se analysis.

intermediate selenol **4** (selenotyrosine), the solution (maintained at pH 6, under nitrogen) was treated with a methanolic solution of methyl iodide to give 4′-(MeSe)-Phe **2,** identified by its characteristic UV spectrum using HPLC diode array analysis.

The stability of 4′-(MeSe)Phe was investigated by storing pure peaks collected off the HPLC column in 65% methanol/0.025% TFA at 4° under air, with or without 1 mM DTT. Slow formation of more polar substances (about 5%) was observed by HPLC analysis over a period of 30 days, and this was partly prevented by DTT.

Synthesis of DL-4′-seleninylphenylalanine: [4′-(SeO_2^-)Phe] (3)

Many seleninic acids have been prepared by oxidation of aromatic selenocyanates or diselenides with concentrated hydrogen peroxide, nitric acid, or peracetic acid.[8] Such aggressive conditions seemed unlikely to be suitable for amino acids, but 3% hydrogen peroxide has been used for selective oxidation of the sulfur in sulfur amino acids and therefore was chosen for oxidation of 4′-(SeCN)Phe **1** to the seleninic acid **3.** Using UV diode array HPLC to monitor the reaction, no oxidation was seen at room temperature over 2 h, but oxidation began to occur at 40° and rapid oxidation ensued upon warming to 80°. The 4′-(SeCN)Phe peak was lost within 15 min, with concurrent formation of a new peak eluted at 5.3 min having a UV absorption peak at 224 nm (not shown). Since seleninic acids are weak acids (pK_a 4–5),[17] anion exchange chromatography was employed for purification of the reaction mixture. The seleninic acid was eluted with 10 mM HCl in 20% methanol (59% yield). Yields were lower when heating at 80° exceeded 15 min, and considerable amounts of strongly retained selenium (not eluted with 0.01 N HCl but eluted with 1 N HCl) were formed, indicating that over-oxidation may have occurred with the longer heating times.

The pooled sample of **3** showed a single ninhydrin- and starch/iodide-positive spot ($R_f = 0.52$ upon TLC in acidic system 1, 0.11 in basic system 2), and anionic mobility upon TLE at pH 7. The charged properties and ninhydrin reactivity confirmed that the product was an amino acid having an additional weak anion moiety; the presence of the seleninic acid functional group was confirmed by the starch/iodide test, which measures oxidation of iodide to iodine and is characteristically given by seleninic acids. At pH 1.6, TLE mobility was cationic, confirming that the anionic groups had been protonated [this weak acid behavior showed that the product is a seleninic acid rather than a selenonic (strong) acid]. Mass spectroscopy and NMR analysis confirmed the structure.

Properties of phenylalanine seleninic acid 3

Reduction of **3** with sodium borohydride (Fig. 3a) generated the spectrum of the selenolate form (λ_{max} 272 nm, $\varepsilon_{mM} = 16.5$) of 4′-(SeH)Phe ('selenotyrosine') **4**. Upon acidification of the sample the UV peak shifted to 244 nm; these spectral properties are similar to those of benzene selenolate (λ_{max} 271 nm, $\varepsilon_{mM} = 13.7$) and benzene selenol.[18] Upon spontaneous oxidation of the borohydride-treated sample, a broad absorption band corresponding to the diselenide **6** was formed (λ_{max} 248 and 329 nm) (Fig. 3b); the spectrum is similar to that of diphenyl diselenide.[18] Electrospray mass spectroscopy of the sample confirmed the formation of the diselenide **6**. The presence of a Cl_8Na_9 ion at $m/z = 488.8$ precluded observation of the diselenide $[M+H]^+$ [^{80}Se] at $m/z = 489.0$ calculated for $C_{18}H_{20}N_2O_4Se_2$, but the expected

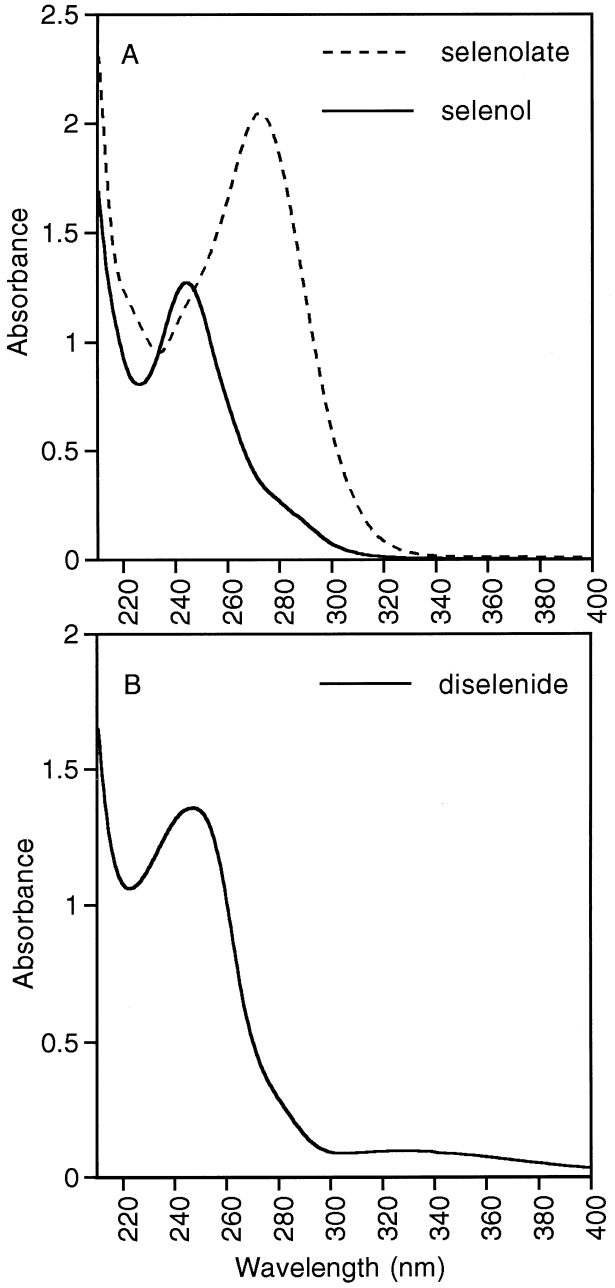


Figure 3. Absorption spectra of selenotyrosine **4**. **A** $NaBH_4$ (50 μL 0.5 M) was added to 0.123 mM 4′-(SeO_2^-)Phe in N_2-sparged water (final volume 2 mL). The spectrum of the borohydride-generated selenotyrosine sample was recorded immediately (dashed line, selenolate) or after acidification to pH 2.2 with 40 μL 1 M HCl (solid line, selenol). **B** Spectrum of selenotyrosine diselenide **6** recorded 16 h after sample (a) was opened to air.

isotope pattern was seen for the doubly-charged diselenide $[M+2H]^{++}$ ion at m/z 245.1.

Addition of methyl iodide to a borohydride-generated sample of 4′-(SeH)Phe gave a new HPLC peak having a UV spectrum and retention time corresponding to the alkylated product **2**. Treatment of a parallel sample of **4** with allyl chloride gave a new HPLC peak (retention time 11.6 min) having properties [UV spectrum and mobility on TLC (R_f = 0.79, system 1)] consistent with the Se-allyl derivative of phenylalanine. Mass spectrometry showed a Se-isotope peak at m/z = 285.9, corresponding to $[M+H]^+$ [^{80}Se] (m/z = 286.0) calculated for (Se-allyl)Phe, $C_{12}H_{15}NO_2Se$.

Synthesis of DL-4′-methylseleninylphenylalanine: [4′(MeSe = O)Phe] (5)

Controlled oxidation of 4′-(MeSe)Phe **2** at 0° using dilute hydrogen peroxide (H_2O_2:Se = 3.3) gave the selenoxide **5**. The reaction was monitored by HPLC, using the disappearance of the 8–9 min peak of **2** as an endpoint; as this peak disappeared a new peak having a maximum at 223 nm was eluted at 5.1 min, along with a trace of hydrogen peroxide. Treatment of an aliquot of this oxidized sample with a slight excess of DTT resulted in restoration of the characteristic peak of 4′-(MeSe)Phe and nearly complete disappearance of the 5.1 min peak, showing that the oxidation was reversible (data not shown). This is consistent with the oxidation product being a selenoxide, rather than some other product such as seleninic acid **3**. Similar results were obtained using oxidation at 4° with an excess of hydrogen peroxide (H_2O_2:Se = 100), followed by addition of excess DTT.

Positive ion mass spectrometry showed one major component corresponding to the protonated molecular ion of the selenoxide **5**. NMR analysis confirmed the presence of a methyl group at 2.84 ppm, compared to 2.36 ppm for the unoxidized parent compound **2**. TLC of the oxidized sample showed a single ninhydrin-positive spot having a low R_f (0.37) compared to the parent selenide (0.77); upon TLE it behaved as a neutral amino acid at pH 7 and had cathodic mobility slightly greater than phenylalanine at pH 1.6. The electrophoretic behavior under acidic conditions is consistent with the slightly basic properties of selenoxides.[8]

Discussion

A new series of Se-substituted phenylalanine derivatives has been synthesized having the para position of the phenyl ring substituted by selenocyanate (-SeCN), seleninic acid (-SeO_2H), or selenol (-SeH) functional groups. Alkylation of the selenol ('selenotyrosine') gave the selenoether derivatives of phenylalanine [(Phe-SeR), R = methyl or allyl], and air oxidation of the selenol gave the diselenide. Mild oxidation of the selenoether 4′-(MeSe)Phe with peroxide gave the selenoxide derivative, 4′-[Se(O)Me]. Six of these compounds were previously unknown; the Se-methyl selenoether 4′-(MeSe)Phe **2** was synthesized previously by a different procedure[19] using addition of methyl Grignard reagent to diethyl acetamido(cyanoselenobenzyl)malonate and subsequent hydrolysis to give the amino acid. The method described here introduces Se into the preformed phenylalanine structure under mild conditions in aqueous solution, avoiding the difficulties associated with Grignard methodology.

Organic selenocyanates and seleninic acids are well known classes of organoselenium compounds with useful properties. The phenylalanine selenocyanate or seleninic acid derivatives described here are stable, water-soluble amino acids with minimal odor. They are suitable for direct use in biological studies, or for introduction of a reactive Se functional group in relatively stable form into peptides or proteins. The phenylalanine seleninic acid is especially convenient as a precursor that can be treated with sodium borohydride or other suitable reducing agent for generating the selenol derivative of phenylalanine, selenotyrosine. The Se-methylphenylalanine selenoether derivative is readily oxidized to give the selenoxide derivative. Selenoxides are far more reactive than their sulfoxide counterparts, and react readily with thiols, leading to possible redox cycling.[20,21] Many aliphatic selenoxides are unstable, whereas alkylaryl selenoxides have good handling properties,[21] thus a phenylalanine-based selenoxide is expected to have superior stability compared to one formed from Se-methylselenocysteine or similar type of aliphatic selenoether.

Use of phenylalanine analogues in biochemistry and therapeutics

Numerous ring-substituted phenylalanine derivatives have been synthesized chemically or occur naturally. Nitro, amino, sulfo, and halogen derivatives were synthesized in the l9th century by Erlenmeyer and other chemists.[10] Other phenylalanine derivatives containing arsenic or sulfur substituents were synthesized for use as antimetabolites.[22,23] The thio analogue of tyrosine ('thiotyrosine') was first synthesized in l912.[9] Escher et al.[24] have described methods for protecting thiotyrosine and other sulfur-containing phenylalanine derivatives for peptide synthesis, and reported some biological activities of angiotensin II analogues containing these amino acids. More recently, aminothiotyrosine disulfide derivatives undergoing facile homolytic cleavage have been used as optical triggers in studies of protein folding kinetics.[25]

Aromatic selenoamino acids have useful properties for a wide range of biochemical applications. The *p*-selenocyanate derivative of phenylalanine is readily prepared and converted to other stable derivatives such as the seleninic acid with standard oxidizing agents. Reduction of phenylalanine-4′-seleninic acid is a convenient means of generating selenotyrosine **4**. Because of the facile oxidation of selenotyrosine, isolation of the free selenol **4** was not attempted, but it can be generated easily for study of redox properties. Alkylation of the freshly-formed selenol using alkyl halides allows synthesis of

Se-substituted phenylalanine analogues. It is hoped that the present study will facilitate further investigations with aromatic selenoamino acids to explore their chemistry and biological significance.

Naturally occurring phenylalanine derivatives

Besides tyrosine, the best known naturally-occurring phenylalanine derivatives are the various iodinated tyrosines and thyronines, and bromo-substituted aromatic amino acids found in marine organisms. The formation of mono- and dibromotyrosine derivatives also occurs in humans through reaction of tyrosine residues with the eosinophil peroxidase that preferentially utilizes bromide or thiocyanate in peroxidative reactions with H_2O_2.[26] It is not known whether analogous metabolic activation reactions might occur with Se, either in intermediary metabolism of Se[4] or in association with its biological function as a component of glutathione peroxidases and other selenoproteins. One purpose of this work was to develop a basis for such studies and synthesize stable model compounds.

Metabolic activation of Se and adduct formation with proteins is known to occur. Methylseleninic acid and inorganic sodium selenite can react with thiol groups of proteins to give adducts containing selenenylsulfide (S−Se) or selenotrisulfide (S−Se−S) bonds.[27] These adducts are cleaved by reducing agents to release Se from the protein. However, there is evidence from radioactive tracer studies[28,29] that proteins may incorporate unidentified forms of Se that are resistant to reductive removal, in addition to selenocysteine and selenomethionine. A mechanism for forming stable Se adducts might be the generation of highly reactive Se intermediates within suitable domains and further reaction with nearby amino acid residues. The successive reaction of two thiol groups with inorganic selenite forms RS-Se(=O)-SR, a very reactive electrophilic intermediate.[30,31] In proteins containing a cluster of two or more proximal cysteine residues, it is conceivable that initial reaction of selenite with two cysteines would be followed by a reaction of this reactive intermediate [RS-Se(=O)-SR] with a nearby residue, forming an aromatic selenoamino acid adduct in the protein. When additional thiol groups are available, further reaction of the RS-Se(=O)-SR intermediate may give the selenotrisulfide RS-Se-SR, plus disulfide, as a competing reaction. A 4:1 thiol/selenite reaction stoichiometry was demonstrated for reduced ribonuclease, a model protein having 8 sulfhydryl groups.[32] In a protein having cysteine residues located in a relatively inaccessible hydrophobic domain, it is possible that the reaction would be constrained to the initial phase that generates electrophilic intermediates from the reaction of only one or two thiol groups, followed by electrophilic substitution of Se onto the ring of tyrosine or phenylalanine.

Some evidence has been obtained for the occurrence of thiolated derivatives of tyrosine in proteins. The active site of galactose oxidase has a novel thioether bond linking Cys 228 and Tyr 272, as shown by crystallographic studies.[33] Substitution by the cysteine sulfur onto the tyrosine ring was suggested to modulate the redox potential of the free radical site in the enzyme.[34] The thioether apparently is attached to tyrosine in the ortho position, but isolation of the thioether bridged dipeptide by chemical methods has not been reported. Phenols undergo facile electrophilic substitution and it is likely that tyrosine would undergo selenylation preferentially compared to phenylalanine. However, substituted phenylalanine derivatives may be more stable than substituted tyrosine derivatives because phenols having Se constituents can undergo oxidative reactions leading to formation of quinones and related products.

Conclusion

The Se-containing phenylalanine derivatives described here can be used in free form, or incorporated into peptides and engineered proteins to increase chemical functionality. The various functional groups are relatively stable because of direct Se bonding to the phenyl ring, and confer physical properties such as polar neutral (-SeCN), hydrophilic anionic ($-SeO_2^-$), or hydrophobic, as in the case of the selenoethers (-SeR). Other modifications of the selenoether derivatives are possible, such as alkylation to give positive-charged selenonium derivatives ($-Se^+R_1R_2$), or reversible oxidation to relatively stable alkylphenyl selenoxides that undergo redox cycling.[21] The selenol group found in selenotyrosine has high reactivity in redox reactions or in nucleophilic reactions. Alkylation of the freshly-formed selenol using alkyl halides allows synthesis of a variety of Se-substituted phenylalanine analogues. Photolability of aromatic selenoamino acid selenocyanates or facile reactions of other Se-containing aromatic selenoamino acids leading to radical formation also could be useful. Anionic Se-substituted analogues of tyrosine are of interest as possible surrogates for phosphotyrosine.

Experimental

Materials and methods

Reagents were obtained from Sigma-Aldrich (St. Louis, MI). Mass spectra were acquired on a SciexAPI365 triple quadrupole mass spectrometer at the University of Wisconsin Biotechnology Center. The instrument was operated in positive ion mode using minimal energy conditions in order to prevent fragmentation of the Se compounds. Samples were injected after acidification with 0.5% acetic acid, or directly injected for samples eluted from ion exchange columns with 0.01 N HCl.

The ^{1}H NMR spectra were obtained at the National Magnetic Resonance Facility in Madison using a Brüker DMX 500 spectrometer. Chemical shifts are reported in parts per million (ppm, δ) relative to DSS [3-(TMS)-1-propanesulfonic acid, sodium salt] as internal standard. Ultraviolet spectra were obtained on a Shimadzu Model 1601 spectrophotometer using 1 cm pathlength quartz cuvettes.

For HPLC a polymer-based reversed phase column (Hamilton PRP-1, 1×10 cm, fitted with a guard column) was operated at 25° using isocratic elution (1 mL/min) with methanol/water (65:35) containing 0.025% trifluoroacetic acid, pH 2.5. A photodiode array detector (Waters Model 991) was used to monitor the UV spectra of eluted compounds. TLC was done on precoated cellulose plastic sheets using system 1 (*n*-butanol/acetic acid:water, 5:2:3, v/v/v) or system 2 (*n*-butanol/pyridine:water, 7:8:6, v/v/v). Ninhydrin (0.2% in acetone) or starch/iodide (1% soluble starch plus 1% KI) were used as detection reagents. Thin layer electrophoresis (TLE) was carried out on 20×20 cm cellulose-coated glass plates at 10°, 350 volts. The buffers used were pH 1.6 (formic acid/acetic acid/water, 150:100:750), pH 5.2 (pyridine/acetic acid/water, 10:2.5:988), or pH 7 (0.05 M HEPES). N-DNP-ethanolamine was used as a neutral marker. Phenylalanine (net charge +1 at pH 1.6) was used as a cationic marker and N-DNP-taurine (net charge -1) as an anionic standard. Selenium was assayed by a fluorometric procedure following acid digestion of duplicate aliquots.[35] Iodometric analysis was done by standard procedures.

Synthesis of compounds. Note: Certain forms of selenium and cyanide are volatile and highly toxic, or have offensive odors. The reactions described should be carried out using apparatus designed to minimize the release of volatile compounds, within a good quality fume hood

DL-4′-(selenocyanato)phenylalanine (1). DL-4′-aminophenylalanine (10 mmol) dissolved in 4.5 mL 6 M HCl was chilled in an ice bath and 5.9 mM $NaNO_2$ in ice-cold water was added in small portions with stirring until starch/iodide test paper gave a positive test (total of 10 mmol $NaNO_2$). The pH was adjusted to 4 by addition of 5 M sodium acetate, and a chilled solution of KSeCN (10.1 mmol in 4 mL water) was added in small portions over 15 min. After evolution of N_2 ceased, the solution (pH about 5) was stirred at room temperature for 19 h. (During this reaction and subsequent operations, care was taken to to protect the sample from light). TLC analysis of the orange-red solution showed loss of the starting compound and the presence of a ninhydrin-positive spot of higher R_f. After dilution and filtration to remove elemental Se, the sample was applied to a large XAD column (230 mL bed vol) previously cleaned as described.[14] After washing with 1 bed vol of water and 100 mL of 20% methanol, 40% methanol was used to elute the nearly pure 4′-(SeCN)-Phe (2.31 mmol, 23% yield). The light-golden solution was adjusted to pH 7.4 and passed through a 2.5×9.5 cm column of Dowex 50 (NH_4^+) to remove small amounts of impurities, giving an almost colorless solution of pure 4′-(SeCN)Phe **1** in 40% methanol. This solution was stored at 4° until used for synthesis of the remaining compounds. A portion was lyophilized to dryness and dissolved in D_2O for NMR. UV: λ_{max} 236 nm ($\varepsilon_{mM}=7.18$), with a shoulder from 250–300 nm. MS: m/z 270.9, $[M+H]^+$ [^{80}Se] calculated 271.0. HPLC retention time 6.3 min. TLC: (system 1) single ninhydrin-positive spot ($R_f=0.7$). TLE: (pH 1.6), single ninhydrin-positive cationic spot, mobility similar to phenylalanine and one-half that of 4′-aminophenylalanine; pH 5.2, single spot, same mobility as phenylalanine. ^{1}H NMR (D_2O) δ 7.76 (d, 2H), 7.37 (d, 2H), 3.99 (m, 1H), 3.14–3.31 (m, 2H).

DL-4′-(methylselenanyl)phenylalanine (2). A solution of 4′-(SeCN)Phe **1** (550 mL, 0.47 mmol) was placed in a 3-necked flask fitted with a pH electrode. Nitrogen was swept through the stirred solution, then a solution of sodium borohydride (2.4 mmol) was added over 30 min, maintaining the pH at 5 to 7 by addition of 1 N HCl. Iodomethane (total of 3.6 mmol) then was added in several portions and the mixture left to react for 22 h at 25° in the dark under N_2. The reaction mixture was concentrated two-fold by rotary evaporation to lower the methanol concentration, then applied to an XAD column. After washing with water followed by 20% methanol to remove salts and other impurities, 4′-(Me-Se)Phe was eluted with 65% methanol, as shown by HPLC analysis of the collected fractions. The leading edge of the peak contained traces of a more polar substance; these fractions were excluded from the final pool of 4′-(MeSe)Phe **2** (55% yield). One half of the pool of pure 4′-(Me-Se)Phe **2** was concentrated to remove the methanol. A portion was lyophilized and dissolved in CD_3OD plus D_2O for NMR analysis. The remainder of the aqueous concentrate, stored at 4°, slowly crystallized. A 65% methanol solution of the crystalline material was scanned to obtain the UV spectrum. UV (65% MeOH): Shoulder 220 nm, ε_{mM}10.4; λ_{max} 251 and 266 nm, λ_{min} 260 nm; ε_{mM} 7.61, 7.32, and 7.09, respectively. MS: m/z 259.9, $[M+H]^+$ [^{80}Se] calc 260.0. HPLC retention time 8.3 min. TLC: (system 1) single ninhydrin-positive spot ($R_f=0.77$). TLE: pH 1.6, single ninhydrin-positive cationic spot. ^{1}H NMR (CD_3OD, D_2O) δ 7.45 (d, 2H), 7.22 (d, 2H), 3.87 (m, 1H), 2.97–3.23 (m, 2H), 2.36 (s, 3H).

DL-4′-(seleninyl)phenylalanine (3). 4′-(SeCN)Phe **1** from the 65% methanol pool off Dowex 50 (20 mL, 0.041 mmol) was concentrated under nitrogen to 1/3 vol. After addition of hydrogen peroxide (3.2 mmol) and warming to 80–85°, HPLC analysis showed complete disappearance of the starting compound within 15 min and formation of a new peak eluted at 5.3 min. The solution was cooled on ice to halt further oxidation. The sample (pH 3.9) was adjusted to pH 7.5 with NH_4OH and applied to a 1.5×7.5 cm Dowex 1 (Cl^-) column previously washed with 3% H_2O_2 and methanol and equilibrated with water. The column was washed with 2 bed vol of water until a negative starch/iodide test was obtained, then elution was begun with 10 mM HCl in 20% methanol. A major UV and starch/iodide positive peak was eluted, followed by a minor second component. The pure fractions containing the main peak of **3** were pooled and analyzed (24 μmols, 59% yield, based on Se analysis and confirmed by iodometric analysis). A 2 mL aliquot was lyophilized and dissolved in 0.5 mL D_2O for NMR. UV (20% MeOH, 0.01 M HCl): λ_{max} 224 nm, $\varepsilon_{mM}=13.8$, with a shoulder from 250 to 280 nm. Mass spectrum: m/z 277.9, $[M+H]^+$ [^{80}Se] calculated 278.0. HPLC retention time 5.3 min. TLC: single ninhydrin- and starch/iodide-positive spot, $R_f=0.46$

(system 1); $R_f = 0.11$ (system 2). TLE: pH 7, single anionic spot; pH 1.6, single cationic spot. ^{1}H NMR (D_2O) δ 7.87 (d, 2H), 7.58 (d, 2H), 4.30 (m, 1H), 3.29–3.46 (m, 2H).

DL-4′-(methylseleninyl)phenylalanine (5). An aliquot of 4′-MeSePhe **4** (10 mmol) was concentrated three fold to remove methanol, then cooled on ice and treated with small portions of H_2O_2 (total of 33 mmol). After 2 h, HPLC showed that the 4′-MeSePhe had completely disappeared, forming a new peak eluted at 5.1 min having an UV maximum at 223 nm. Treatment of an aliquot of the reaction mixture (0.083 mmol Se) with DTT (0.5 mmol) caused loss of the 5.1 min peak, with reappearance of the peak of 4′-MeSePhe, followed by a peak of oxidized DTT (λ_{max} 282 nm) at 11.1 min. An aliquot of the H_2O_2-oxidized 4′-(MeSe)Phe sample was lyophilized to dryness and dissolved in D_2O to give a solution of **5** for NMR analysis. UV (65% MeOH, 0.025% TFA): λ_{max} 223 nm, shoulder 255–280 nm. MS: m/z 275.9, $[M+H]^+$ [^{80}Se] calc 276.0. HPLC retention time 5.1 min. TLC: (system 1) single ninhydrin- and starch/iodide-positive spot ($R_f = 0.37$). TLE: pH 1.6, single ninhydrin- and starch/iodide-positive cationic spot, slightly higher mobility than phenylalanine; pH 7, single neutral spot (same mobility as phenylalanine). ^{1}H NMR (D_2O) δ 7.77 (d, 2H), 7.54 (d, 2H), 4.02 (t, 1H), 3.22–3.34 (m, 2H), 2.85 (s, 3H).

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