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The Reaction of *p*-Tolyl Isoselenocyanate with Primary and Secondary Amines: A Multinuclear Magnetic Resonance Study[1]

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Isoselenocyanates are known to react with primary and secondary amines to produce the corresponding selenoureas. *p*-Tolyl isoselenocyanate was prepared and its reaction with various primary and secondary amines was investigated. The resulting selenoureas were characterized by multinuclear (^{13}C and ^{77}Se) magnetic resonance spectroscopy. Since these selenoureas possess a C=Se bond, the ^{77}Se chemical shifts display a high sensitivity to the chemical and electronic environment of the nucleus and a large range for relatively minor changes in chemical structure. The rationale for the choice of *p*-tolyl isoselenocyanate is that it clearly resembles, in both structure and reactivity, the corresponding, well-studied, phenyl isothiocyanate (Edman's reagent), which has widespread applicability in the sequencing of amino acid residues in polypeptides and proteins.

INTRODUCTION

Selenium-77 has several properties which make this nucleus an appealing candidates as an NMR probe for organic and biochemical systems (*2*). The natural abundance of ^{77}Se is 7.5% and this isotope has a nuclear spin $I = \frac{1}{2}$. The wide chemical shift range of ^{77}Se (~3000 ppm) renders this nucleus extremely sensitive to its electronic environment, a property which is particularly well-suited for NMR investigations of macromolecular systems. The fact that selenium is known to serve as an essential micronutrient and that its presence is required for the function of certain selenoproteins and seleno-tRNAs has heightened interest in the application of ^{77}Se NMR spectroscopy to biochemical systems (*3, 4*).

Previously, we synthesized the selenium-containing reagent, 6,6′-diselenobis(3-nitrobenzoic acid) which was designed to chemically modify sulfhydryl groups in proteins (*5*). This reagent in the presence of biological sulfhydryl groups afforded the corresponding selenenyl sulfide derivatives of reduced and denatured ribonuclease and lysozyme, thereby demonstrating the feasibility of observing selenium resonances in these modified macromolecules (*6, 7*). Subsequently, various selenium-containing inhibitors and substrates were constructed and their interactions with the enzyme, α-chymotrypsin, were characterized by both kinetics and ^{77}Se NMR spectroscopy (*8, 9*). Our success in observing relatively large changes in ^{77}Se chemical shifts in ligands bound, either covalently or noncovalently, to pro-

[1] Taken in part from Ref. (*1*).

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teins has motivated us to extend our investigations to the incorporation of selenium into biochemical molecules by both synthetic and biosynthetic routes.

One of our approaches to synthetic incorporation is to construct selenium-containing analogs of well-known protein chemical modification agents. We have chosen to synthesize and study a selenium-containing analog of phenyl isothiocyanate, commonly known as Edman's reagent, which is widely known for its utility in the amino acid sequence analysis of proteins and peptides (*10*). Additionally, various isothiocyanates have been used as affinity labels (*11*), hydrophilic membrane labels (*12*), and specific irreversible enzyme inhibitors (*13, 14*).

The choice of investigating a selenium analog of Edman's reagent was further motivated by the fact that the chemistry of isothiocyanates and isoselenocyanates is similar in that they both react with primary and secondary amines to produce the corresponding thioureas and selenoureas, respectively (*15*). Therefore, it should be possible to extend the Edman chemistry used in amino acid sequencing to a comparable isoselenocyanate and produce the appropriate selenourea, whose subsequent cyclization leads to the corresponding hydantoin. A further impetus for this study was that ^{77}Se NMR investigations have demonstrated that compounds containing a C=Se moiety possess the largest chemical shift range (>2600 ppm) of any class of selenium-containing compounds (*1*) and ^{77}Se chemical shifts for only a few selenoureas have been reported (*16, 17*). We report the synthesis, characterization, and ^{13}C and ^{77}Se NMR studies of *p*-tolyl isoselenocyanate and a series of corresponding selenoureas.

EXPERIMENTAL

Spectral and Analytical Data

Selenium-77 NMR spectra were obtained in the Fourier transform mode on Bruker WP200 or WH400 superconducting spectrometers (38.16 and 76.31 MHz, respectively). Chemical shifts were measured with respect to $(CH_3)_2Se$ (60% in $CDCl_3$) (*18*). Positive chemical shifts denote resonances that are deshielded with respect to the reference. Measurements were typically made at, or near, ambient probe temperature in 15- or 10-mm NMR tubes using $CDCl_3$ as an internal lock solvent. All spectra were acquired in the proton-coupled mode; generally, 0.3 M solutions were used and 5000–10,000 scans were acquired using a pulse angle of 30° and a recycle time of 2.2 s. A resolution of 0.1 ppm was obtained using an 8K data table and a sweep width of 19,000 Hz.

Carbon-13 spectra were obtained in 5-mm tubes on an IBM NR-80B spectrometer (20.11 MHz) operating in the Fourier transform mode employing continuous broadband noise-modulated proton decoupling. Chemical shifts are reported with respect to tetramethylsilane with a positive value denoting a resonance deshielded from the reference. Measurements were made at ambient probe temperature using $CDCl_3$ as an internal lock solvent with concentrations of approximately 0.3 M. Generally, 4000–7500 scans were acquired to achieve sufficient signal to noise.

Melting points were recorded from a Mel-Temp melting point apparatus and are reported uncorrected. Elemental analyses were performed by Galbraith Laborato-

ries, Inc. (Knoxville, TN). Ultraviolet data were acquired on a Hewlett–Packard 8450A uv–visible spectrophotometer; all samples were dissolved in absolute ethanol. Mass spectra were measured on a Finnigan 4021 GC/MS instrument and a VG 70SQ spectrometer.

Synthesis

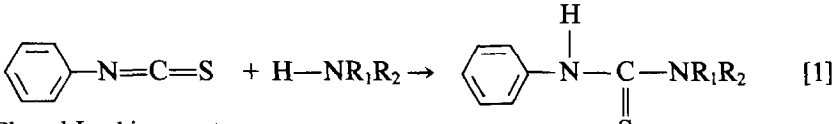
The following compounds were prepared by known methods: *p*-tolyl isoselenocyanate; *N*-phenyl-*N'*-*p*-tolylselenourea; *N,N'*-bis(*p*-tolyl)selenourea; *N*-*o*-tolyl-*N'*-*p*-tolylselenourea (*19*).

Preparation of selenoureas from p-tolyl isoselenocyanate: A general procedure. In a typical experiment, 1 g (5 mmol) of *p*-tolyl isoselenocyanate was dissolved in 100 ml of absolute ethanol. The solution was stirred and 5 mmol of the appropriate primary or secondary amine was added over the next 30 min. During the course of the reaction, a constant nitrogen atmosphere was maintained in the reaction vessel. The solution was heated gently (ca. 40°C) for 1 h for the reaction with primary amines and approximately 12 h for the reaction with secondary amines. The products were isolated as solids and recrystallized from ethanol. It was necessary to store all materials in amber bottles to limit the amount of decomposition since these materials appeared to be light sensitive. It should also be noted that these compounds all appeared to be thermally stable and were maintained on the shelf for months without any decomposition.

RESULTS AND DISCUSSION

In this investigation we have synthesized and characterized *p*-tolyl isoselenocyanate and its reaction with a variety of amines to produce the corresponding selenoureas. In earlier studies, the existence of isoselenocyanates was assumed since amines could be added *in situ* to the presumed compound to produce the expected selenoureas (*20, 21*). Since that time, the synthesis and characterization of isoselenocyanates have been reported by several investigators (*22–27*). Infrared (*28*) and ^{1}H NMR studies (*29*) have been reported but ^{13}C and ^{77}Se data currently are lacking in the literature.

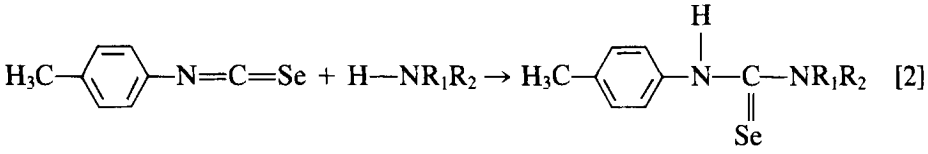
Phenyl isoselenocyanate, the selenium analog of Edman's reagent (phenyl isothiocyanate), is an acrid oil that is air sensitive. It has recently been utilized by Iskierko and co-workers (*30*) to identify the N-terminal amino acid residues of β-insulin. On the other hand, *p*-tolyl isoselenocyanate is a crystalline solid that is air and moisture stable. *p*-Tolyl isoselenocyanate was chosen for this study because it is similar in structure and reactivity (Eqs. [1] and [2]) to, but easier and safer to handle, than phenyl isoselenocyanate:



[1]

Phenyl Isothiocyanate
Edman's Reagent

Thiourea



[2]

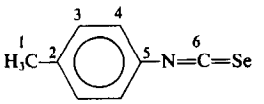
p-Tolyl Isoselenocyanate Selenourea

p-Tolyl isoselenocyanate was prepared as described previously (*31*) and from this compound and the appropriate amine, seven selenourea derivatives, containing both alkyl and aromatic substituents, were synthesized. Carbon-13 and ^{77}Se NMR spectral data are provided in Table 1. Carbon-13 assignments were generally straightforward; however, some difficulty was encountered in assignment of the aromatic carbon resonances; therefore, ring carbon assignments were done with the help of additivity substituent values (*32*). In the selenoureas, which have the general formula *p*-$(CH_3)C_6H_4N(H)C(=Se)NR_1R_2$, the ^{13}C resonances were unaffected by the choice of R_1 or R_2. For example, the resonance of the methyl group *para* to the aromatic ring (carbon 1; see Table 1 for numbering system) is measured to be 21.0 (±0.1) ppm in all compounds. There is a larger influence exhibited toward the aromatic resonances of carbons 2–5, but the range for these ^{13}C resonances is no larger than 2.7 ppm for any particular carbon. The ^{13}C resonance of the carbon bound to selenium (carbon 6) shows the largest sensitivity to the choice of R_1 or R_2 which is easily explained by the closer proximity of this carbon to these groups. This resonance varies from 176.1 (compound **4**) to 181.4 ppm (compound **3**). It is of interest to note that in the parent isoselenocyanate (compound **1**), no ^{13}C resonance was observed for carbon 6 under a variety of experimental conditions (Fig. 1). These included extended relaxation delays of 5 s and total recycle times of up to 7 s. A similar phenomenon is observed for the analogous sulfur compound, *p*-tolyl isothiocyanate. It has been reported previously (*33*) that for a series of *para*-substituted phenyl isothiocyanates, including the *para*-methyl case, great difficulty was encountered in detecting the isothiocyanate carbon in the ^{13}C NMR spectrum. Only at a high molar concentration of the compound was the resonance detected and the accuracy of these values was questioned by the authors. They attributed this situation to characteristic quadrupolar broadening (from the adjacent nitrogen) and, thus, along with ineffective spin-lattice (T_1) relaxation experienced by the carbon nucleus, presumably accounted for the difficulty encountered in the detection of the isothiocyanate (and isoselenocyanate) resonance. More recently, the ^{13}C NMR spectrum of phenyl isothiocyanate, ^{13}C enriched at the isothiocyanate carbon, was reported (*34*) and exhibited a very broad resonance at 135.95 ppm which was attributed to the isothiocyanate carbon.

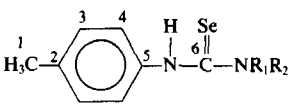
As noted previously, only a few ^{77}Se NMR chemical shifts for selenoureas have been reported to date (*16, 17*). One feature evident from these shifts is the enormous sensitivity to rather small changes in the chemical and electronic environment of the selenium nucleus. For example, if we compare the ^{77}Se chemical shift of $H_2NC(=Se)NH_2$ (195 ppm) (*16*) with the ^{77}Se chemical shift of $H_2NC(=Se)NMe_2$ (147 ppm) (*17*), a change of almost 50 ppm occurs. This is significant

TABLE 1

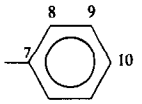
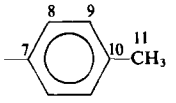
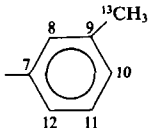
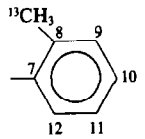
Selenium-77 and Carbon-13 NMR Chemical Shifts of *para*-Tolylisoselenocyanate (**1**) and Corresponding Selenoureas (**2–8**)



1



2–8

Compound	R_1	R_2	δ^{77}Se (ppm)[a]	1	2	3	4	5	δ^{13}C (ppm)	6	7	8	9	10	11	12	13
1	—	—	−300	21.2	138.4	130.1	125.8	127.0		*[b]							
2	$^7CH_2{}^8CH_3$	$^7CH_2{}^8CH_3$	239	21.0	137.9	129.3	126.7	136.3		180.7	47.3	12.4					
3	$^7CH(^8CH_3)_2$	$^7CH(^8CH_3)_2$	241	21.0	137.9	129.2	127.2	136.4		181.4	51.4	20.4					
4	$^7C(^8CH_3)_3$	H	258	20.9	137.5	130.7	125.2	133.6		176.1	54.9	29.1					
5		H	245	21.0	137.5	130.3	125.7	134.5		178.7	137.8	125.6	129.5	127.4			
6		H	245	21.0	136.7	130.3	125.6	134.9		179.0	134.9	125.7	130.3	136.7	21.0		
7		H	228	21.0	137.5	130.0	125.6	134.8		179.1	137.2	126.1	139.6	128.2	129.3	122.6	18.0
8		H	239	21.0	137.6	130.1	125.9	134.8		178.6	135.9	135.3	131.3	128.5	127.9	127.1	21.2

[a] Selenium-77 chemical shifts are relative to 60% Me_2Se in $CDCl_3$.
[b] Not observed; see Results and Discussion.

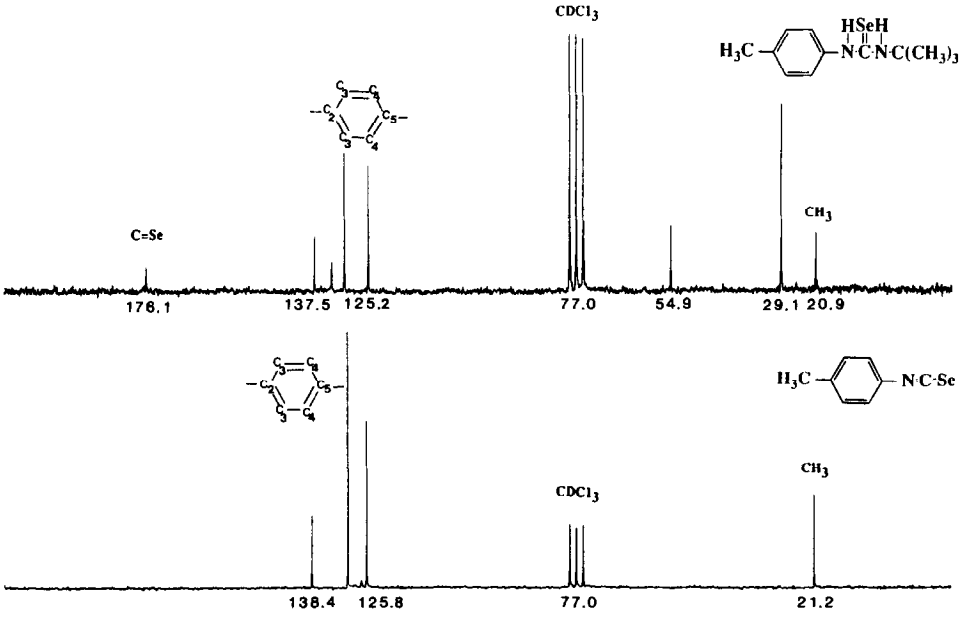


FIG. 1. Carbon-13 NMR spectra of *p*-tolyl isoselenocyanate (compound **1**) and *N*-*t*-butyl-*N'*-*p*-tolylselenourea (compound **4**). Note that no ^{13}C resonance is observed for carbon bound to selenium in *p*-tolyl isoselenocyanate.

considering the fact that two protons are being replaced by two methyl groups three bonds removed from the selenium nucleus.

The ^{77}Se chemical shifts of the compounds studied in this investigation also clearly demonstrate the large change observed in the resonance frequency for this nucleus. The parent isoselenocyanate (compound **1**) has a ^{77}Se resonance which is shielded over 500 ppm from the resulting selenoureas. Among the selenoureas themselves, there is a smaller, but still significant, change of 30 ppm with the most deshielded resonance resulting in compound **4** (where R_1 = *t*-butyl and R_2 = H) and the most shielded resonance arising in compound **7** (where R_1 = *m*-tolyl and R_2 = H). It is interesting to note that the addition of a methyl group in the *meta* position (compound **7**) shields the ^{77}Se resonance by 17 ppm with respect to the unsubstituted compound (compound **5**), whereas a methyl group in the *para* position (compound **6**) causes no ^{77}Se chemical shift change with respect to compound **5**.

Ultraviolet spectral data are given in Table 2. The ultraviolet spectrum of *p*-tolyl isoselenocyanate has been previously reported (*35*) and our spectrum compares favorably with that reported. All compounds (**1–8**) exhibit strong molar absorptivities and when comparing the spectrum of *p*-tolyl isoselenocyanate with those of the seven selenoureas, several features are apparent. First, upon formation of a selenourea from the isoselenocyanate, the absorbances at 235 and 293 nm disappear. Additionally, new absorbances are observed at 227 and 269 nm for compounds **2** and **3** and at 288 nm for compound **4**. In compounds **5–8** (where two

TABLE 2

Ultraviolet Data on *para*-Tolyl Isoselenocyanate (**1**) and Corresponding Selenoureas (**2–8**)

Compound	A_{max} (nm)	Molar extinction coefficient (m^{-1} cm^{-1})
1	210	36,800
	235	53,300
	293	17,400
2	207	15,600
	227	17,000
	269	9,000
3	208	19,500
	227	18,300
	269	9,900
4	206	13,400
	288	7,600
5	205	28,500
	282	13,500
6	202	22,000
	282	11,050
7	207	26,000
	286	12,100
8	204	35,700
	281	12,000

aromatic rings are present), an absorbance in the 280-nm range is seen. Also, all compounds possess a strong absorbance between 202 and 210 nm.

Mass spectroscopy was used in the initial identification of all compounds. This technique is especially useful in the detection of selenium-containing compounds since selenium has numerous isotopes that have detectable natural abundances. These isotopes are ^{74}Se (1%), ^{76}Se (9%), ^{77}Se (8%), ^{78}Se (24%), ^{80}Se (49%), and ^{82}Se (9%) and thus, in compounds that possess selenium, a characteristic selenium "fingerprint" pattern is observed. This fingerprint disappears in fragments that no longer contain selenium and allows for easy interpretation of mass spectral data. A general fragmentation pattern where the entire molecular ion is seen followed by selenium loss is observed in every case. Other characteristic fragments generally seen are *m/e* 91 (indicative of Me-C_6H_4) and *m/e* 107 (indicative of Me-C_6H_4-NH).

In conclusion, this study is the initial investigation of *p*-tolyl isoselenocyanate as a potential agent in our continuing studies of biological systems containing selenium. The next logical step would be to extend this chemistry to amino acids and more complex biological compounds, such as proteins. These represent potential areas that can be studied using *p*-tolyl isoselenocyanate and multinuclear magnetic resonance spectroscopy. These results demonstrate again the advantages of using ^{77}Se NMR spectroscopy in detecting small nuances in the chemical and electronic environment of the selenium nucleus. This sensitivity is even

greater in compounds containing carbon–selenium double bonds and we hope to exploit this sensitivity in our continuing investigations of biological systems that contain selenium via multinuclear magnetic resonance spectroscopy.

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