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Transformation of a wobble 2-thiouridine to 2-selenouridine via *S*-geranyl-2-thiouridine as a possible cellular pathway

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ABSTRACT

The newly discovered *S*-geranylated 2-thiouridines (geS2U) (Dumelin et al., 2012) and 2-selenouridines (Se2U) were recently shown to be synthesized by a single enzyme (selenouridine synthase, SelU) through two distinct pathways using the same 2-thiouridine substrate (S2U); however, no clear catalytic mechanism was proposed. We suggest that *S*-geranyl-2-thiouridine is an intermediate of the SelU-catalyzed conversion of S2U to Se2U. The successful chemical transformation of S2U → geS2U → Se2U is demonstrated here as an initial approximation of the intracellular pathway. The structure of Se2U was confirmed by spectroscopic methods, which included, for the first time, ^{77}Se NMR data (δ 354 ppm).

1. Introduction

Transfer RNAs (tRNAs) are unique among natural RNAs because of their substantial content of modified nucleosides, including 5-substituted uridines (x5U, **1**) and their analogs containing either a sulfur (2-thiouridines, x5S2U, **2**) or selenium atom (2-selenouridines, x5Se2U, **3**) in the nucleobase C2 position. These hypermodified nucleosides are located in the wobble position of the anticodon (position 34) of tRNAs specific for glutamine, glutamic acid and lysine [1,2]. Their structural features play a fundamental role in the tuning of codon reading and the efficiency of the translation process [3]. Ten type **2** 2-thiouridines and five type **3** 2-selenouridines are known; the substituents at C5 (x) are shown in Fig. 1.

Two hydrophobic 5-substituted-2-thiouridines in which the sulfur atom is functionalized with a geranyl residue (mnm5geS2U, **4d** and cmnm5geS2U, **4e**) were recently identified in several bacterial strains at position 34 of tRNAs specific for lysine, glutamine and glutamic acid [4]. tRNA geranylation is induced by cellular stress and occurs in less than 7% of the cell population. The geranylation of the wobble S2U nucleoside affects the codon preference and frame shifting during translation. Although the exact mechanism of this effect is unknown, the superior recognition of the G-ending over the A-ending codons by geranylated tRNA^{Lys} is observed.

Selenouridine synthase SelU is the enzyme responsible for selenoation of 2-thiouridine in *Escherichia coli* [5], and Liu and co-workers have demonstrated that this enzyme is also involved in the geranylation of 2-thiouridine [4]. These two chemically disparate processes employ the same 2-thiouridine substrate. For selenoation, the sulfur atom in 2-thiouracil must be converted into a leaving group and further substituted by a selenium nucleophile originating from selenophosphate. By contrast, for geranylation, the same sulfur atom acts as a nucleophile and accepts the geranyl substituent from geranyl diphosphate. It is unclear how these two mechanistically different reactions are mediated by the same catalyst. Moreover, the potential relationship between the two distinct products, Se2U and geS2U, formed from the same precursor is also of interest.

The literature data suggests that the chemical transformation of S2U to various C2-substituted uridines might occur through the respective *S*-alkylated derivatives. For example, *S*-alkylation of 2-thiouridine yielded a series of 2-alkyl(aryl)aminouridines [6]. In addition, *S*-methylated 2′-deoxy-2-thiouridine was successfully transformed (via a hydrazino derivative) into a 2-azidonucleoside [7]. Huang et al. demonstrated that *S*-methylated 2-thiouridine is efficiently transformed to 2-selenouridine upon nucleophilic attack of the hydroselenide ion (HSe^-) on the electropositive C2 center [8]. Accordingly, we hypothesized that the newly discovered

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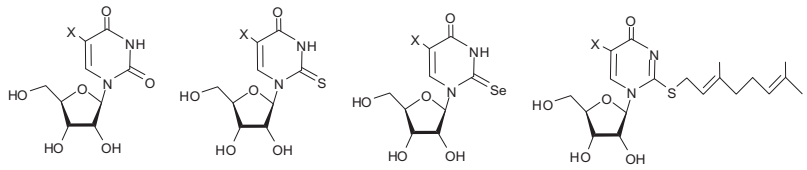


Substituent x	Notation for x	2	3	4
—H		a	a	a
—CH_3	m	b	-	-
—CH_2NH_2	nm	c	c	-
—CH_2NHCH_3	mnm	d	d	d
—CH_2NHCH_2COOH	cmnm	e	e	e
—$CH_2NHCH_2CH_2SO_3$	τm	f	-	-
—$CH_2NHCH_2CH{=}C(CH_3)_2$	inm	g	-	-
—CH_2COOH	cm	h	-	-
—CH_2COOCH_3	mcm	i	-	-
—CHO	f	j	j	-

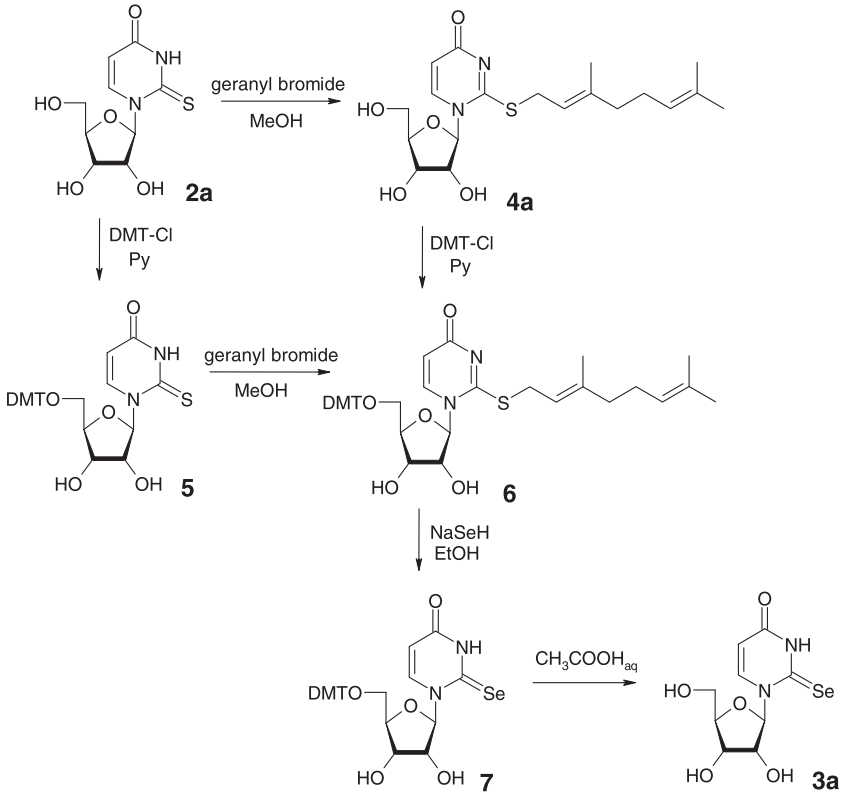
Fig. 1. Structures of the known 5-substituted uridines (x5U, **1**), 2-thiouridines (x5S2U, **2**), 2-selenouridines (x5Se2U, **3**) and *S*-geranyl-2-thiouridines (x5geS2U, **4**).

S-geranyl-2-thiouridine is an intermediate formed during the transformation of 2-thiouridine (embedded in a tRNA chain) to 2-selenouridine. We therefore attempted the chemical transformation S2U → geS2U → Se2U as an initial approximation of the intracellular mechanism.

2. Results

The *S*-geranylated 5′-*O*-DMT-2-thiouridine (**6**) was obtained from 2-thiouridine (**2a**) either through its geranylated form **4a** [4] and subsequent dimethoxytritylation at the 5′-OH function or via 5′-OH protection with a DMT group, yielding compound **5,** and subsequent geranylation of the sulfur residue (Scheme 1). The structure of *S*-geranyl-2-thiouridine **4a** was confirmed by ^{1}H and ^{13}C NMR and FAB-MS spectral analysis (for numbering, see Supplementary Material Fig. S2; for spectral analysis, see Figs. S3–S5). The 5′-DMT protection of **4a** was accomplished using standard protocols to give pure **6**. A similar approach was used for geranylation after 5′-OH protection (**2a** → **5** → **6**) (see the Supplementary Material for preparative procedures and ^{1}H NMR spectral data of **5**, Figs. S6 and S7). The efficiencies of the first and second synthetic approaches were similar and produced pure derivative **6** in 66 and 46% yield, respectively. The structure of **6** was confirmed by ^{1}H and ^{13}C NMR and FAB mass spectrometry (Figs. S8–S13). The DMT group improved the solubility of **6** in organic solvents, thus facilitating the subsequent selenoation.

The sodium hydroselenide (NaHSe) required for the subsequent reaction was prepared according to the published procedure [8] by



Scheme 1. Transformation of S2U **2a** to Se2U **3a** via an intermediate geS2U **6**.

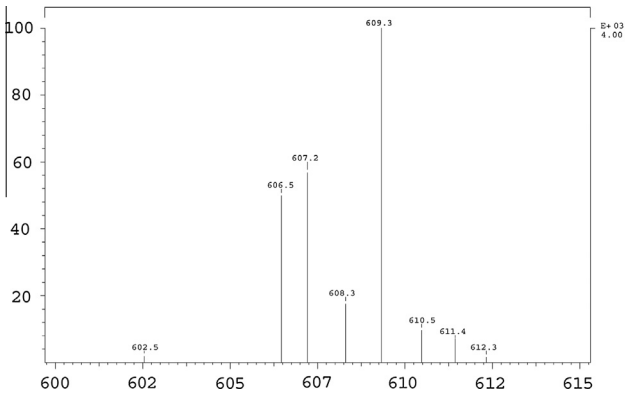


Fig. 2. FAB MS spectrum for **7**. Signals at *m*/*z* 609.3 and *m*/*z* 606.5 correspond to the molecules containing the most abundant ^{80}Se isotope and to those artificially enriched in ^{77}Se isotope, respectively.

reduction of elemental selenium with $NaBH_4$ in absolute ethanol under an inert atmosphere. Importantly, the selenium used in the experiment contained 22 at.% of the NMR-active ^{77}Se isotope, in contrast to the natural abundance of ^{77}Se isotope, which is approximately 7.6%. A solution of ^{77}Se-enriched hydroselenide was added to **6**, and the mixture was stirred for 6 h under an inert atmosphere. After aqueous work up, silica gel flash chromatography furnished **7** as a yellow solid in 30% yield.

The structure of **7** was confirmed by ^{1}H and ^{13}C NMR and by FAB mass spectrometry (Figs. S14–S17). The 22% abundance of ^{77}Se retained in **7** was documented by an extended FAB MS spectrum (Fig. 2, *m*/*z* 606.5). The resonance line of ^{77}Se in the labeled uridine (Fig. 3A, 77Se NMR, δ 407.4 ppm) was located downfield of the signal for ^{77}Se-selenourea (δ 195 ppm) [9]. ^{77}Se enrichment permitted the acquisition of a 2D HMBC spectrum (Fig. 3B), in which the signal for H1′ at δ 6.5 ppm was correlated with the ^{77}Se signal at δ 407.4 ppm.

Compound **7** was then detritylated with aqueous acetic acid (50%) for 1 h at room temperature. The resulting product **3a** (Se2U) was isolated by RP-HPLC and characterized by UV (λ_{max} 301 nm), ^{1}H and ^{13}C NMR (in D_2O), and FAB MS (Figs. S18–S23). The ^{77}Se isotope contents in **3a** and **7** were identical (Fig. 2 and S24). The ^{77}Se NMR resonance signal for **3a** was observed at δ 353.7 ppm (Fig. S25), i.e., it was shifted approximately 54 ppm upfield from the ^{77}Se resonance of **7**.

3. Discussion

In this study, we demonstrate that the chemical transformation of S2U to Se2U proceeds easily via the *S*-geranylated S2U intermediate, in which substitution at the C2 position with nucleophilic selenide occurs, accompanied by the departure of the thioalkyl moiety. Under cellular conditions, this reaction is strictly dependent on the concentration of the selenium donor. At low selenium concentrations (<10 nM), the geranylated tRNA form is predominant over the seleno-tRNA form; however, selenoation occurs at the expense of the formation of geranylated tRNA at selenium concentrations exceeding 10 nM [4]. Thus, this transformation seems to occur in the cell in a linear manner via S2U → geS2U → Se2U (Scheme 2) rather than in a parallel manner in which the two individual reactions S2U → Se2U and S2U → geS2U occur independently, possibly in different catalytic centers [4,5].

The source of selenium in the S2U to Se2U enzymatic transformation is a selenophosphate ($SePO_3^{3-}$), which is the product of the conversion of selenide (HSe^-) by selenophosphate synthetase (SelD) [10,11]. The reaction utilizes ATP and yields $SePO_3^{3-}$, orthophosphate and AMP. Selenophosphate is also an essential substrate for the generation of selenocysteine (Sec), which is now considered a 21st amino acid. However, selenocysteine is not formed by the direct exchange of sulfur with selenium in cysteine but from $tRNA^{Sec}$ charged with serine [12,13]. The biosynthesis of Sec begins with phosphorylation of the serine moiety catalyzed by phosphoseryl-tRNA kinase (PSTK) and yields *O*-phosphoseryl-$tRNA^{Sec}$ [14]. Activated in this way, the hydroxyl group of serine is replaced by selenium from selenophosphate to afford Sec-$tRNA^{Sec}$. This process supports our hypothesis because attack by the selenium donor is accompanied by the departure of a negatively charged phosphate.

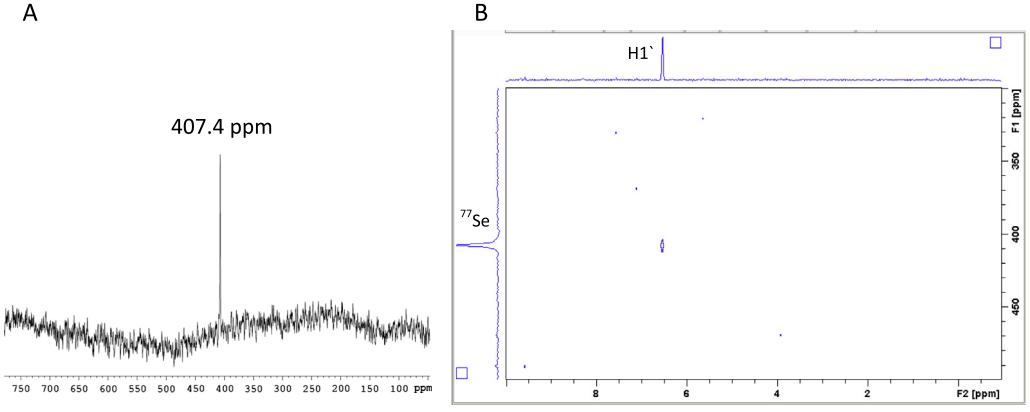
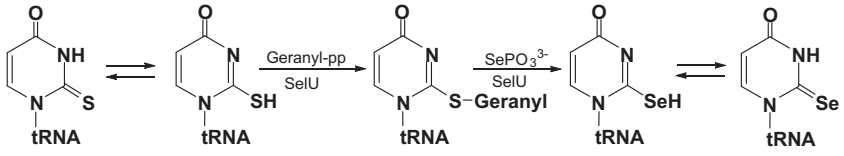


Fig. 3. The ^{77}Se NMR spectrum of 5′-*O*-DMT-2-selenouridine **7** (A); the two-dimensional HMBC spectrum showing a cross-peak between H1′ at δ 6.5 ppm and ^{77}Se signals at δ (B); ^{77}Se NMR (δ 407.4 ppm) for spectrum taken with Ph–Se–Se–Ph reference (δ 463 ppm) in $CDCl_3$.



Scheme 2. Scheme of a possible cellular pathway of S2U-tRNA to Se2U-tRNA transformation undergoing through the geS2U-tRNA intermediate (Geranyl-pp – geranyl diphosphate, SelU-selenouridine synthase).

From the other side, the naturally occurring rare nucleoside *S*-methyl-2-thioadenosine (2-methylthioadenosine, mS2A) is not the product of consecutive sulfuration and methylation of adenosine moiety but of a direct transfer of the methylthio group from SAM [15]. In contrast to the discussed modification there is no any adenosine derivative which would be formed by a nucleophilic attack at C2 of adenine ring of mS2A with subsequent departure of thiomethyl leaving group. Thus, more advanced biological studies are required to evaluate the hypothesis that the transformation of 2-thiouridine to 2-selenouridine follows the linear process S2U → geS2U → Se2U.

4. Conclusions

The successful chemical synthesis of Se2U from S2U via *S*-geranyl-2-thiouridine presented in this study suggests a possible mechanism of SelU enzyme action in which geS2U serves as an intermediate product of the cellular transformation of S2U to Se2U.

5. Experimental

5.1. Synthesis of *S*-geranyl-2-thiouridine (geS2U, **4a**) [4]

2-Thiouridine (260.0 mg, 1 mmol) was dissolved in methanol (10 ml). To the solution geranyl bromide (570 μl, 3 mmol) and *N*,*N*-diisopropylethylamine (522 μl, 3 mmol) were added. After stirring at 25 °C for 30 min the reaction mixture was concentrated *in vacuo*. The residue was dissolved in ethyl acetate and washed with water. The organic layer was dried over anhydrous $MgSO_4$, concentrated *in vacuo* and subjected to silica gel chromatography (0–5% methanol in ethyl acetate) to yield a white solid (355.0 mg, 0.89 mmol, 89% yield): TLC *Rf* = 0.10 (1/9 MeOH/EtOAc (*v*/*v*)); ^{1}H NMR (700 MHz, $CDCl_3$) δ 8.21 (d, *J* = 7.6 Hz, 1H), 5.96 (d, *J* = 7.6 Hz, 1H), 5.91 (d, *J* = 5.7 Hz, 1H), 5.28 (t, *J* = 8.1 Hz, 1H), 5.06 (t, *J* = 7.0 Hz, 1H), 4.45 (q, *J* = 5.2 Hz, 1H), 4.36 (dd, *J* = 5.2, 2.5 Hz, 1H), 4.19 (q, *J* = 2.5 Hz, 1H), 3.93 (m, 2H), 3.87–3.81 (m, 2H), 2.10 (m, 2H), 2.02 (m, 2H), 1.73 (s, 3H), 1.69 (s, 3H), 1.60 (s, 3H). ^{13}C NMR (176 MHz, DMSO-d6) δ 166.6, 161.9, 141.1, 139.4, 131.0, 123.7, 117.4, 108.9, 91.3, 85.9, 74.6, 69.9, 60.7, 29.5, 25.9, 25.4, 17.6, 16.1. MS-FAB (*m*/*z*): 397.3 $[M + H]^+$, MW: 396.5.

5.2. Synthesis of 5′-*O*-dimetoxytrityl-*S*-geranyl-2-thiouridine (**6**) from **4a**

A solution of *S*-geranyl-2-thiouridine **4a** (355.0 mg, 0.89 mmol) and 4,4′-dimethoxytrityl chloride (603.0 mg, 1.78 mmol) in pyridine (4 ml) was stirred at 25 °C for 20 h. To the resulting reaction mixture, 10 ml water was added and extracted with CH_2Cl_2. Organic layer was dried over anhydrous $MgSO_4$, concentrated *in vacuo* and subjected to silica gel chromatography (0–2% methanol in methylene chloride with 0.5% Py). A white solid (470.0 mg, 0.67 mmol, 75% yield) of **6** was obtained after column chromatography: TLC *Rf* = 0.67 (1/9 MeOH/$CHCl_3$ (*v*/*v*)); ^{1}H NMR (700 MHz, $CDCl_3$) δ 7.84 (d, *J* = 7.7 Hz, 1H), 7.43 (dd, *J* = 8.3, 1.4 Hz, 2H), 7.38–7.16 (m, 13H), 6.91–6.86 (m, 4H), 5.92 (d, *J* = 6.3 Hz, 1H), 5.57 (d, *J* = 7.7 Hz, 1H), 5.32 (m, 1H), 5.09 (m, 1H), 4.66 (q, *J* = 5.6 Hz, 1H), 4.43 (m, 1H), 4.26 (q, *J* = 2.6 Hz, 1H), 3.98 (dd, *J* = 12.8, 8.0 Hz, 1H), 3.87 (dd, *J* = 12.8, 8.0 Hz, 1H), 3.79 (s, 6H), 3.45 (t, *J* = 2.8 Hz, 2H), 2.18–2.08 (m, 2H), 2.09–1.98 (m, 2H), 1.76 (s, 3H), 1.71 (s, 3H), 1.63 (s, 3H); ^{13}C NMR (176 MHz, $CDCl_3$) δ 171.20, 166.70, 161.08, 146.69, 145,40, 142.29, 137.46, 134.26, 132.54, 131.41, 130.61, 129.45, 127.68, 126.07, 118.44, 115.75, 111.14, 93.98, 89.53, 87.10, 77.10, 74.25, 65.91, 57.58, 42.05, 34.15, 28.79, 28.08, 20.11, 18.91. MS-FAB (*m*/*z*): 699.50 $[M + H]^+$, MW: 698.88.

5.3. Synthesis of 5′-*O*-dimetoxytrityl-2-selenouridine (**7**) [8]

Nucleoside **6** (80 mg), $NaBH_4$ and Se were dried overnight under reduced pressure. A solution of NaSeH was generated by addition of absolute ethanol (5 ml) to selenium (110 mg of selenium of natural abundance and 23 mg of ^{77}Se) and sodium borohydride ($NaBH_4$, 94 mg) at 0 °C. The reaction was completed in 1 h and clear solution was formed. The ethanolic solution was added to **6** (80 mg) and the mixture was stirred for 3 h under argon. The reaction mixture was concentrated under reduced pressure and chloroform (5 ml) was added to the residue. The organic layer was washed twice with water (2 × 3 ml), and then dried over anhydrous magnesium sulfate. Purification was performed by flash chromatography (4% methanol in methylene chloride) and the light yellow compound **7** was obtained (30% yield). TLC *Rf* = 0.60 (1/9 MeOH/$CHCl_3$ (*v*/*v*)); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.61 (s, 1H), 8.26 (d, *J* = 8.1 Hz, 1H), 7.67 (t, *J* = 7.6 Hz, 1H), 7.38–7.31 (m, 2H), 7.31–7.16 (m, 10H), 6.85–6.76 (m, 4H), 6.55 (d, *J* = 1.5 Hz, 1H), 5.58 (d, *J* = 8.1 Hz, 1H), 4.49 (dd, *J* = 7.7, 5.3 Hz, 1H), 4.44 (dd, *J* = 5.3, 1.5 Hz, 1H), 4.19 (dt, *J* = 7.7, 2.3 Hz, 1H), 3.76 (d, *J* = 1.9 Hz, 13H), 3.56 (qd, *J* = 11.3, 2.3 Hz, 2H), 1.29–1.16 (m, 5H). ^{13}C NMR (126 MHz, Chloroform-d) δ 175.64, 159.40, 158.72, 149.50, 144.29, 140.73, 136.43, 135.22, 134.97, 130.14, 128.10, 127.24, 123.94, 113.35, 108.14, 96.64, 87.13, 84.05, 68.81, 61.03, 55.29. ^{77}Se NMR (95 MHz, $CDCl_3$) δ 407.45. MS-FAB (*m*/*z*): 609.30 $[M + H]^+$, MW: 608.53.

5.4. Synthesis of 2-selenouridine (**3a**)

2-Selenouridine (**3a**) was prepared through detritylation of 5′-*O*-dimethoxytrityl-2-selenouridine **7** (5 mg, 0.008 mmol) by acid treatment (1 h, room temperature) (quantitative removal of DMT group). Then the reaction mixture was concentrated under reduced pressure (SpeedVac) and the remaining crude 2-selenouridine was purified by RP-HPLC. The product elution was performed with a gradient of buffer A (20 mM triethylammonium acetate TEAAc, pH 7.1 in water) to buffer B (20 mM triethylammonium acetate TEAAc, pH = 7.1 in 50% acetonitrile) on a Kinetex C18 column (5 μm, 4.6 mm × 250 mm, Phenomenex) at a constant flow rate of 1.0 ml/min (0 → 15 min from 0% to 25% of B; 15 → 20 min from 25% to 100% of B). Yield 1.95 mg (ca. 80%). ^{1}H NMR (600 MHz, D_2O) δ 8.03 (dd, *J* = 8.1, 1.3 Hz, 1H), 6.61 (d, *J* = 2.5 Hz, 1H), 6.14 (d, *J* = 8.1 Hz, 1H), 4.29 (q, *J* = 2.5 Hz, 1H), 4.07 (m, 2H), 3.88 (dd, *J* = 13.1, 1.6 Hz, 1H), 3.73 (dd, *J* = 13.1, 2.5 Hz, 1H). ^{13}C NMR (151 MHz, D_2O) δ 175.58, 162.83, 141.52, 108.04, 95.86, 83.97, 74.92, 68.33, 59.74. ^{77}Se NMR (95 MHz, D_2O) δ 353.79. MS-FAB (*m*/*z*): 307.2 $[M + H]^+$, MW: 306.16.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.05.012.

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