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Article

The Enigma of Sponge-Derived Terpenoid Isothiocyanate-Thiocyanate Pairs. A Proposal for their Biosynthesis

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Abstract: The co-occurrence of rare terpenoid thiocyanates (R-C-SCN), structurally similar to their more common isothiocyanate isomers (R-NCS) remained an enigma that can now be rationalized by consideration of three integrated biosynthetic motifs: terpenoid carbocation capture by cyanoformate, NC-COOH (itself in equilibrium with NC^- and CO_2), a co-localized rhodanese (a dual-function enzyme that can both convert inorganic NC^- to thiocyanate ion, NCS^-, and alkyl isonitriles to alkyl isothiocyanate (R-NC –> R–NCS). This scenario explains the preponderance of isothiocyanates, R-NCS as products of a linear reaction path – α-addition of S^0 to R-NC – over the minor, less stable thiocyanates, R-SCN, as products of adventitious capture of liberated NCS^- by the penultimate terpenoid carbocation precursor. DFT calculations support the proposal and eliminate other possibilities, e.g. isomerization of R-NCS to R-SCN.

Keywords: Alkyl isothiocyanate; alkyl thiocyanate; farnesyl pyrophosphate; geranylgeranyl pyrophosphate; malaria; marine natural product; nudibranch; Porifera; isonitrile; rhodanese; thiocyanate

1. Introduction

Exotic terpenoid isonitriles (**TI**s, **Figure 1**) which occur exclusively within the domain of certain genera of marine sponges (Porifera), e.g. *Acanthella*, *Adocia*, *Axinella*, Axynissa, *Cymbastella* among others, and the seaslugs (nudibranchs) that prey upon them, have been known since the early 1970s. Marine isonitriles and their derivatives have been extensively reviewed [1,2,3]. Examples of cyclized terpenoid isonitriles and their accompanying α-adducts (**1a-c**, **2a–l**, **3**) are depicted in **Figure 2**. Only recently have certain members (e.g. 7,20-diisocyanoadociane (**3**) [4], kahilinols A (**1a**) [5] and B (**1b**) [6]) been identified and investigated as potential antimalarial therapeutics. Isonitrile **1b** exhibits potent activity against the chloroquine-resistant plasmodium, Dd2 (IC_{50} = 4.6 nM). MED6-189 (**2**), a synthetic analog developed from **1b**, shows promising in vitro antiplasmodial activity (Dd2 IC_{50} = 47± 7 nM) and strong efficacy in plasmodium-infected mice with no apparent toxicity or hemolytic liabilities [7].

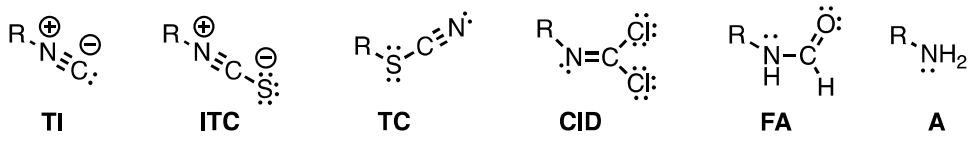

Figure 1. Lewis structures (closed shell) of terpene isonitriles (**TI**s), and isothiocyanates (**ITC**s) and thiocyanates (**TC**), carbonimidic dichlorides (**CID**), formamides (**FA**) and amines (**A**).

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TIs are often accompanied by the corresponding adducts generated by α-addition to the triple -NC bond; amides, carbonimidic dichlorides, and, most commonly, alkyl isothiocyanates [8]. Sponge-derived isonitrile biosynthesis can be rationalized by capture of late-stage terpenoid carbocations arising from C-C cyclizations and Wagner-Meerwein rearrangements of their activated precursors (GPP, FPP and GGPP) and final capture by a biosynthetic 'NC vector' [9] to generate isonitriles, R-NC. Isothiocyanates, R-NCS, e.g. kalihinol M **1d**) (**Figure 2**) and **2a** follow as the products of sulfur-transfer reaction of low-valent S donors (e.g. glutathione dimer, thiosulfate, etc.) to R-NC. Terpenoid carbon backbones of **TIs** and related derivatives, including isothiocyanates (**ITC**, **Figure 1**) are mostly familiar in plant terpenoids, but notable exceptions are synthesized only by sponges. For example, the common plant sesquiterpene (C_{15}) skeletons of cadinane, drimane, and bisabolane are all represented in the sponge **TI**, **ITC** and **TC** family, but sponges and their predators (nudibranchs) are the exclusive provenance of axinyssane [10,11], pupukeanane [12,13], neopupukeanane and the diterpenes (C_{20}) adociane, [4], amphilectane [14,15], isoneoamphilectane [16] among others.

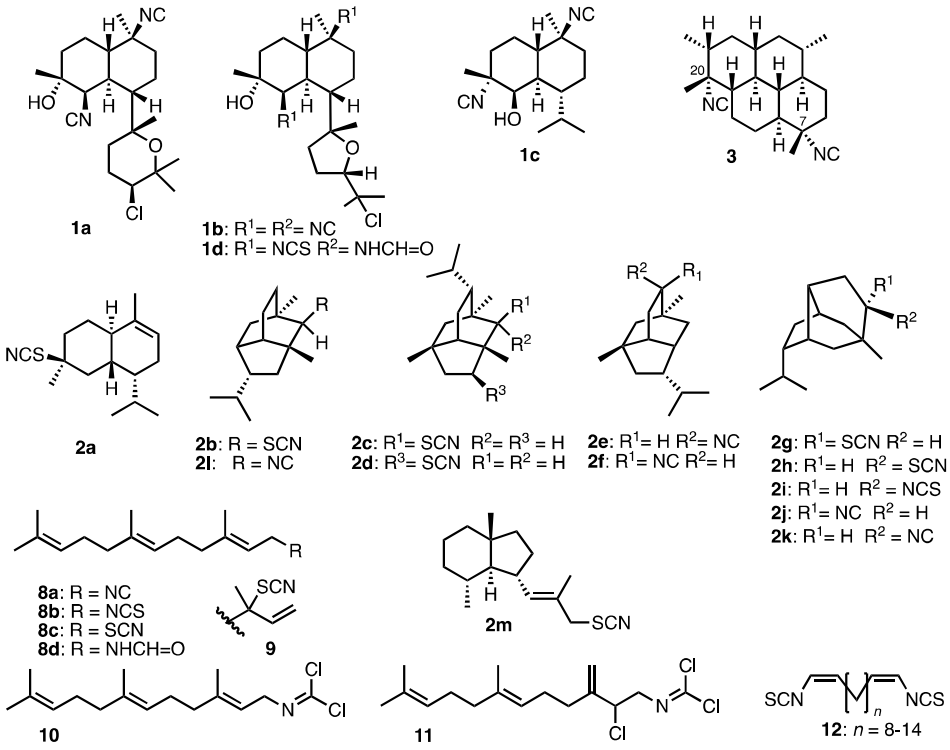

Figure 2.) Terpenoid isonitriles (**TI**), related isothiocyanates (**ITC**s) and thiocyanates (**TC**) including antimalarial kalihinol B (**1d**) and synthetic MED6-189 (**1c**).

The startling discovery of co-localized thiocyanates (R-SCN), e.g. 4-thiocyanato-9-cadinene **2a** [17], **2b** [18] (with the same carbon skeleton as isonitrile **2j** [12]), **2c-d** [19]**,** with carbon skeletons similar to those of the more common isothiocyanates (R-NCS), **2g,h** [20], and **2m** [21], presents an enigma [22]: 'how can a linear biosynthetic pathway of R-NC –> R–NCS accommodate R–SCN?' While the biosynthesis of allyl thiocyanate, the pungent volatile natural product found in mustard oil, horseradish, garlic and many other cruciferous plants and its isomerized regioisomer, allyl *iso*thiocyanate, has long-been understood (decomposition of the glucosinolate, sinigrin, derived from Met [23]), the origin of sponge-derived thiocyanates is puzzling. Now, this enigma is addressed by consideration of a putative interplay of three integrated biosynthetic sequelae: capture of the terpenoid carbocation R^+ by cyanoformate, NC-COOH (***i***) [9] (itself in equilibrium with NC^- and CO_2 and derived from Gly), reactions promoted by co-localized rhodanese – a dual-function enzyme that can both convert inorganic NC^- to thiocyanate ion, NCS^-, and alkyl isonitriles to alkyl isothiocyanate

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(R-NC –> R–NCS) – and promiscuous terminal interception of terpenoid carbocations by co-liberated inorganic NCS^-.

2. Results and Discussion.

2.1. Chemistry and Bonding in TC and ITC.

While hundreds of marine-derived **TI**s, **ITC**s and **FAs** have been described., only six **TC**s are known. The structures of the **TC**s were examined on a case-by-case basis, with their isomeric or similar isothiocyanates (**ITC**). 'Reverse engineering' of the likely terminal steps in their biosynthesis by probing the thermodynamics of bond-breaking and bond-forming provides insights into what could be possible and what is improbable. For the purposes of informed discussion, a brief review of the chemistry of the cumulated-bond functional group, NCS, in **TC** and **ITC** molecules is in order. **TC** and **ITC** isomers are easily differentiated spectroscopically by ^{13}C NMR and IR. **ITC** shows a *very* intense, broad stretching frequency at ~2100 cm^{-1}, while the **TC** IR band is of similar frequency, but narrow and of medium intensity. The ^{13}C NMR signal of C at the point of attachment, reflects the difference in electronegativity of S and N: **ITC** shows C-N_α δ ~ 75 while the C-S_α signal in **TC** is δ ~ 64 ppm [22].

The isomers *t*-butylthiocyanate (**4**) and *t*-butylisothiocyanate (**5**) (**Figure 3**) serve as convenient models for comparisons to their terpenoid natural product counterparts. Bonding in the triatomic grouping NCS differs between **TC** and **ITC**. DFT calculations of the optimized geometries and energies of each (**Figure 1**) shows, as expected, the geometry of N-C-S is linear due to sp hybridization at C (ω = 179.8 ° and ω = 179.2°, respectively), isoelectronic with alkyl azides, consistent with X-ray crystal structures of natural product **ITC**s [24] and **TC**s [17]. The bondings of the heteroatom attached to the alkyl group C (notated here as the α atom) differ significantly. The corresponding bond angles and bond lengths of **4** and **5** (θ(C-S-C) = 100° and (θ(C-N_α- C) = 176.8°) reflect predominantly sp^3 and sp hybridization, respectively, of the α-heteroatom attached to the *t*-Bu group. The shorter C-N_α bond length in **5** (d_2 = 1.44 Å is evident of a stronger σ bond between the *t*-Bu group and its α-heteroatom compared to **4** (d_1 = 1.88 Å), as expected for row 2 versus a row 3 elements, and recapitulates the thermodynamic stability of **5** over **4** [25].

ITCs are electrophilic: like isonitriles they undergo addition reactions at the cumulated bond, even with weak nucleophiles. **TC**s reluctantly undergo nucleophilic substitution reactions at the attached C when it 1° or 2°, but not 3°. Both allylic **ITC**s and **TC**s may participate in [3,3]-sigmatropic rearrangement (see below).

Three scenarios that may account for generation of **TC** natural products are considered.

2.2. Dissociation-Reassociation.

The isomerization of **TC** to **ITC** was examined as a possible linear pathway linking the corresponding TI (R-NC –> R–NCS –> R-SCN). Of necessity, such an isomerization must follow a unimolecular rate law, $R = k$[R-NCS] and an S_N1 mechanism due to the 3° alkyl group that is almost invariably the point of heteroatom attachment in **TC** and **TCI** natural products. The leaving group ability of the thiocyano group in R-SCN is at best weak, while in R-NCS, it is considerably poorer due to the stronger R-N bond. For various reasons, the bond dissociation energy in each of the two isomers is not readily calculated, but we can obtain a qualitiative picture from the relative the rates of sigmatropic [3,3] rearrangements of isomeric allylic thiocyanate (e.g. **6**) to isothiocyanates (e.g. **7**) and the isoelectronic allyl azides (e.g. **8**, **Figure 4**). The equilibrium constants, K_{eq}, of the reactions quantitatively reflect their relative stabilities. While the reaction rates of [3,3] rearrangement of allylic azides (Winstein rearrangement [26]) are fast (most rapidly isomerize at ambient temperatures [27]), CH_2=CH-NCS and CH_2=CH-SCN interchange more slowly [28]. For example, the isomerization of the latter to the former occurs upon distillation (b.p. 150-2 °C) [29]) and is favored for reasons of greater bond-strength in the product (vide supra) [30]. The likely explanation of the slower rate

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reaction of [3,3]-rearrangement of **ITC**-**TC** pairs is poor overlap of row 2 versus row 3 frontier orbitals in the transition state.

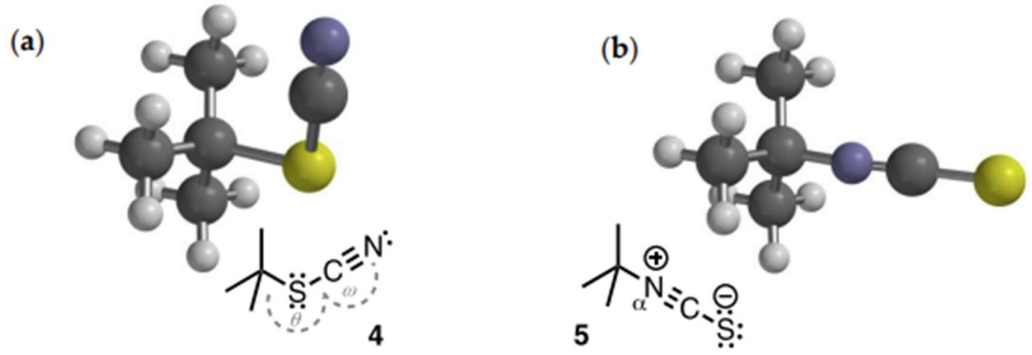

Figure 3. DFT optimized molecular structures (ωB97X-D 6-31G*), dipole moments, key bond angles, and distances, *d* of isomeric **TC** and **ITC**. (a) *t*-butylthiocyanate (**4**): μ = 4.48 D θ_{thio}(C-S-C)= 100.0°, d_{thio}(C–S)= 1.88 Å, and (b) *t*-butylisothiocyanate (**5**): μ = 6.56 D, θ_{iso}(C–N$_\alpha$–C)= 176.8°, d_{iso}(C-N$_\alpha$) = 1.44 Å. The major Lewis closed shell resonance forms are depicted.

2.3. Precedence Strengthens the Role of Adventitious NCS⁻

ITCs are expressed by bacteria from isonitriles by task-specific adapted rhodanese. Rhodanese is distributed widely in the Nature and carries out the important role of scavenging inorganic cyanide formed adventitiously in metabolism, e.g. 'leakage' from C-1 tetrahydrofolate metabolism or other cyanide-generating reactions [31,32,33]. A key intermediate, cyanoformate, NC-COOH, **CF**, *is* ephemeral in the biosynthesis of the plant hormone ethylene, CH_2=CH_2 from 1-aminocyclopropane-1-carboxylate [34]. In *Burkholderia gladioli* that produces the non-terpenoid isonitrile, Hertwick and coworkers showed that rhodanese RhDE, in addition to donating competency in scavenging cyanide NC^- into thiocyanate, NCS^-, catalyzes the substrate-specific sulfur transfer reaction R-NC –> R-NCS generating the **ITC**, sinapigladioside [35], by utilizing thiosulfate as an S donor [36]. Hertwick speculates that the enzyme was recruited from a, "ubiquitous detoxification enzyme for the formation of a bioactive specialized metabolite" [36]. The concept of recruitment of rhodanese, with evolved substrate-specificity to the latter task, may also find support in the biosynthesis of terpenoid **ITC**s in sponges, and the observation that **TI**s are not uniformly accompanied by their **ITC** counterparts.

2.4. Thiocyanate is a competent ambident nucleophile.

Pearson and coworkers compiled relative rates of nucleophilic substitutions of methyl iodide, CH_3I, with selected nucleophiles under comparable conditions (25 °C) [37]. The second-order rate constant, k_2, for reaction with NCS^- and NC^- (25 °C, Table 1) are similar (Entries 8 and 9: 5.74 x10^{-4} and 6.5 x 10^{-4} $mol^{-1}.sec^{-1}$, respectively (for comparison, k_2 for the reactions with thiophenoxide PhS^-, thiosulfate, $S_2O_3^{2-}$ are 1.07 and 0.114 $mol^{-1}.sec^{-1}$ [37]).

A simplified scenario can be proposed for the terminal step in **TC** and **ITC** biosynthesis (**Figure 5**). S_N1 capture of NCS– at S (the more nucleophilic end) gives **TC** (*path a*). **ITC** is formed by S_N1 capture at N which contributes to the pool of **ITC** generated by S addition to the corresponding isonitrile, **TI**. Experimentally, it would be difficult to estimate the fraction of **ITC** that arises from *paths b* and *c*. As mentioned earlier, the results of radiolabeling experiments by NCS^- are somewhat equivocal and at this point do not lend insight into the contributions of *paths b* and *c*.

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Table 1. Second-order rate constant (k_2, S_N2) of CH_3-I with selected nucleophiles (25 °C) (Pearson [37]).

Entry	Nu: or Nu:⁻	$10^3.k_2$ /$M^{-1}.s^{-1}$	Entry	Nu: or Nu:⁻	$10^3.k_2$ /$M^{-1}.s^{-1}$
1	MeOH	1.3×10^{-7}	7	PhO^-	0.073
2	NH_3	0.041	8	NCS^-	0.574
3	N_3^-	0.078	9	NC^-	0.645
4	Br–	0.0798	10	$NCSe^-$	9.13
5	I^-	3.42	11	PhS^-	1070
6	$(CH_3)_2S$	0.045	12	$S_2O_3^{2-}$	114

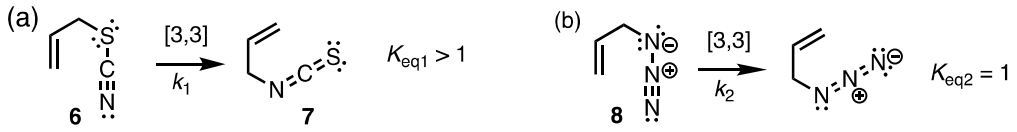

Figure 4. Isomerization of allylic pseudohalides, C_3H_5--NCS and C_3H_5-N_3.(a) Sigmatropic [3,3]-rearrangements: (a) allyl thiocyanate (**6**) to allyl isothiocyanate (**7**). (b) Degenerate rearrangement of allyl azide (**8**).

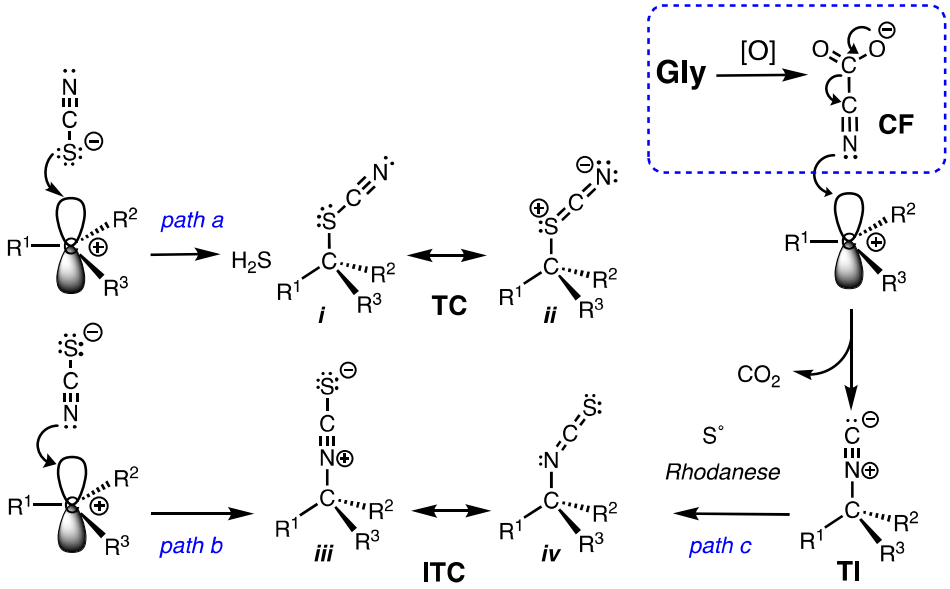

Figure 5. Mechanism of formation of **TC** (path a), **ITC** and **TI**.

Inspection of the structures of secondary metabolites containing the R-SCN group (**TC**s) excludes the pre-described enzyme system for tailoring of R-NC to R-CNS, but still supports a role of rhodanese as a generator of the ambident nucleophile NCS^-. **TC**s may arise from adventitious capture of thiocyanate at S by S_N1 addition, a reaction that would also add to the pool of **ITC** from the former mechanism (Scheme 1).

The well-known rule of thumb for ambident nucleophilicity holds that the bond-forming reaction occurs at the less negative atom, e.g. enolate reactions with most electrophiles occur at the α-C center instead of O. The rule predicts **TC**s are kinetically favored over **ITC**s when NCS^- reacts with electrophiles, R-X (X= halogen) at S, but typically mixtures of **TC**s and **ITC**s are formed. It should be remembered that an extrapolation of the same reaction, but with replacement of $NCS^-$with NC^- gives *only* nitriles, R-CN, not isonitriles, by exclusive nucleophilic addition at C [9]. While HCN is a weak Brønsted acid (pKa = 9.25), HSCN is strong (pKa = –0.7 [38]). HSCN is fully ionized at

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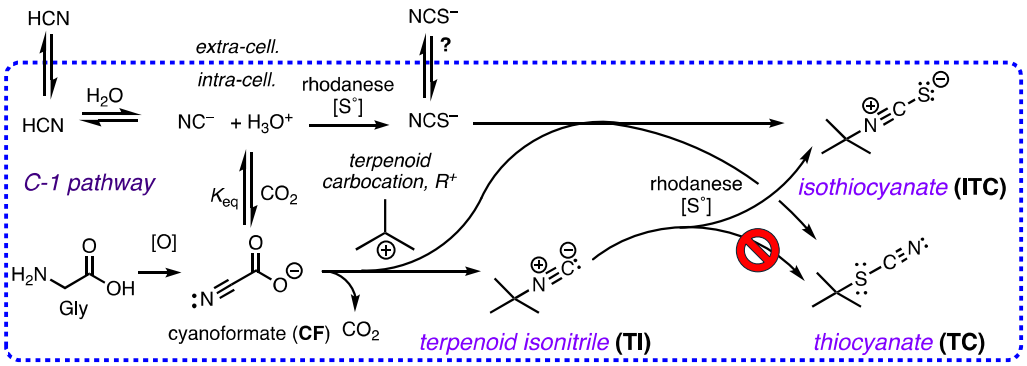
physiological pH. In short, NCS^- is more an 'equal opportunity' nucleophile than NC^- . At equilibrium, isomerization would favor **ITC** over **TC**.

2.5. A Simple Proposal – Thiocyanates Arise Adventitiously.

How do these foregoing data affect interpretation of the provenance and distribution of alkyl isothiocyanate, ITC, versus alkyl thiocyanate, TC? Alkyl TCs are rarer than their isomeric ITCs. Biosynthetically, ITCs mostly arise from a linear pathway of TI –> ITC by addition of low-valent sulfur, the less abundant TCs more likely arise from adventitious nucleophilic capture of free thiocyanate, NCS^- (delivered by rhodanese) by terpenoid carbocations in a parallel pathway (Scheme 1). A convincing appreciation of this proposition is gained by analysis of reported isolation yields of TCs relative to their analogous conspecific ITCs from the same organism. Like the substitution products of NCS^- with electrophilic carbocationic precursors, ITCs are found to predominate over TCs. One remaining question is how can exogenous NCS^- assimilated by sponges that make TC-ITC pairs? While exogenous cyanide can intercept the intracellular dynamic equilibrium of cyanoformate (CF, NC-COOH) by passive diffusion in its neutral form, HCN, the pK_a of HSCN would seem prohibitively low (–0.7) for a similar process; a 'thiocyanate ion transporter' would need to be invoked [39].*Thiohalobacter* sp., with induced capacity to metabolize NCS^-, have been raised by repeated passage in NCS^- containing culture medium [40].



Scheme 1. Unified proposal for biosynthesis of sponge terpene isonitrile (**TI**), isothiocyanate (**ITC**) and thiocyanate (**TC**) natural products.

In each case where analogous **TC** and **ITC** pairs do co-occur, the structures of the terpenoid carbon skeletons differ; **2g,h** and **2i** are the exceptions [20]. An interpretation of this phenomenon is that the last step of S_N1 capture to form **TI** or **TC** is rate-dependent upon the structure of the incipient carbocation, R^+ and determined by its collapse and capture of a NC delivery 'vector', as recently proposed (**Figure 4**, [9]) or, in the latter case, free NCS^-. If R^+ is more persistent (longer half-life), the S_N1 capture by 'off-pathway' NCS^- becomes more competitive, leading to product distributions favorable to **TC**.

Natural product allylic thiocyanates, e.g. farnesyl isothiocyanate and thiocyanate (**8b,c**, **Figure 1**), hypothetically obtainable from nucleophilic substitution of farnesyl pyrophosphate, or **8b** separately by S-transfer to farnesyl isonitrile (**8a**, which itself, "remains elusive" [1]), are kinetically labile. **TC**s in time would be expected isomerize to their more substituted *tert*-allylic NCS counterparts (e.g. **9**). The majority of sponge-derived **TC**s, however, are 'fixed'; stabilized by substitution at 3° alkyl groups. While farnesyl isothiocyanate (**8b**) [41] and formamide, **8d**, are known [42], but the foregoing reasons make it unlikely the 'missing' **8c** (or **8a**?) will ever be isolated from natural sources as [3,3]-rearrangement irreversibly converts **8c** to its isomeric nerolidyl isothiocyanate isomer, **9**. The fact that **9** has not been found in Nature suggests that **8c**, too, is absent. From the single literature report of syntheses of **8c** and its geranyl homolog, the isolated products were invariably

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found mixed with **9** (**8c**:**9**~ 83:17) even after mild vacuum distillation conditions [43,44]. It should be noted the names 'stylotellane A' and 'stylotellane B' were conferred upon the carbonimidic dichloride **10**, and the related **11** [10], from *Styletta aurantium* [45. The latter natural product was originally isolated by Wratten and Faulkner from *Pseudaxinyssa pitys* – the first example of this functional group in a natural product.[10] – along with its chloro-axinyssane analog, **12**. Compounds **8b,d** and **10** complete the set of known farnesyl N-derivatives. It appears that **2m** is the only *allylic* **TC** among these marine natural products, with the NCS group substituted at the 'tail' of the first isoprene group in the precursor FPP. The unexpected, unbranched long-chain lipids, **13**, from *Pseudoaxinyssa*, containing vinyl-substituted α,ω-bisisocyanato groups, it can be noted, appear to have a different, indeterminate, biosynthesis [46].

One consequence of a strictly unimolecular reaction of NCS^- with R^+ when the product creates a new asymmetric center at the electrophilic C, are diastereomeric mixtures. The product distribution will be dependent upon the usual factors that govern stereofacial preferences of S_N1 reactions, e.g. steric hindrance and stereoelectronic factors. For example, in the case of epimeric **2g** and **2h**, isolated from the sponge *Axinyssa aculeata* [20], the reported ratio is 3:2 [47], but from local symmetry considerations the two epimers should be about equal in terms of G°.

When thought of in this way, capture of carbocations by free NCS^-, is a 'clock reaction'; a kinetic monitor of the partition of the shunt reaction of cyanoformate, **CF** (NC-COOH Scheme 1)– dissociation to NC^- and CO_2 – and direct nucleophilic S_N1 capture by NCS^- when the ratios of epimers can be measured. Garson [11,41,48] and others [49] have shown through numerous radiolabeling experiments that inorganic $[^{14}C]CN^-$ is assimilated into sponge **TI**s. For example, 7,20-diisocyanoadociane (**3**) is radiolabeled by $[^{14}C]CN^-$ [50]. These observations have recently been interpreted as an interception of an equilibrium cyanide pool formed by dissociation of **CF**, the putative 'NC' vector, and exogenous uptaken HCN [9]. In the same study, Garson found no radiolabel incorporation into **3** when the sponge was incubated with [2-^{14}C] Gly or a number of other amino acids [51,52].

The prevailing belief up to now was that inorganic NC^- is the precursor of **TI**s, but for several reasons this was shown to be untenable [9]. If it were so, a simple test can be made: the expected labeling of living sponges with $[^{14}C]$-CN^- should also induce formation of limiting amounts of R-SCN from rhodanese-promoted conversion to NCS^- and the kinetic-product distribution of nucleophilic capture, but this has not been observed to date [53]. Garson and coworkers showed that radiolabeled thiocyanate (K[^{14}N]NCS) is assimilated by living sponges into **TI**s including axisonitrile-3 (notably with a formula lacking S) and two **ITC**s (axisothiocyanate-3) albeit with far lower specific radioactivities than the same radiolabeled natural products obtained from incubations with Na{^{14}C]CN [54]. Simultaneous labeling of axisonitrile-3 from incubation of the sponge *Acanthella cavernosa* [55] with K[^{14}N]NCS requires, at the very least, an unspecified interchange of thiocyanate with K[^{14}C]CN [^{56}]. In a separate study, Simpson and Garson found incorporation of both $[^{14}C]SCN^-$ and $[^{14}C]CN^-$ into the sesquiterpene thiocyanato group of **2b**[18,19], from *Axinyssa* sp. n. from the Great Barrier Reef [57]; both results are consistent with the adventitious thiocyanate model for **TC** biosynthesis (**Figure 4, Scheme 1**)

What could be testable in the field is incorporation of N^{14}C-COOH in sponges producing **TI**s, **ITC**s and **TC**s or N$^{14}CS^-$ under more controlled conditions. The logistics of this proposed experiment are beyond the scope of this report, but the outcomes would, nevertheless, be compelling.

3. Materials and Methods

3.1. . General Experimental Procedures.

DFT Calculations. All DFT calculations were performed using Spartan '20 (Wavefunction, Irvine, CA) using functional ωB97X-D and basis set 6-31G* (H_2O or gas phase). Coordinates for the optimized structures and bond parameters can be found in the Supporting Information. See Supporting Information for complete citation to the DFT product.

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4. Conclusions

A proposal is forwarded for the likely origins of the rare sponge-derived terpenoid thiocyanates (**TC**s) that are decoupled from the biosynthesis of isothiocyanates (**ITC**s). Isothiocyanates (**ITC**s) likely arise from a low-valent sulfur-transfer reaction to precursor terpenoid isonitriles (**TI**s). Adventitious thiocyanate ion, NCS^-, a competent amphiphilic nucleophile generated by rhodanese, is solely responsible for sponge **TC** terpenoid natural products in two ways; S_N1 addition at S produces **TC**s. Addition of rhodanese-derived NCS^-, at N to the incipient terpenyl carbocation R^+, contributes to the **ITC** pool by simultaneous converse S_N1 reaction.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. The following supporting information can be downloaded at: www.mdpi.com/xxx/s1 DFT calculated structures of **4**, **5** and 1-isothiocyanato-1-methylcyclohexane (**S1**).

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Conflicts of Interest: The author declares no conflict of interest.

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