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Residual Complexity Does Impact Organic Chemistry and Drug Discovery: The Case of Rufomyazine and Rufomycin

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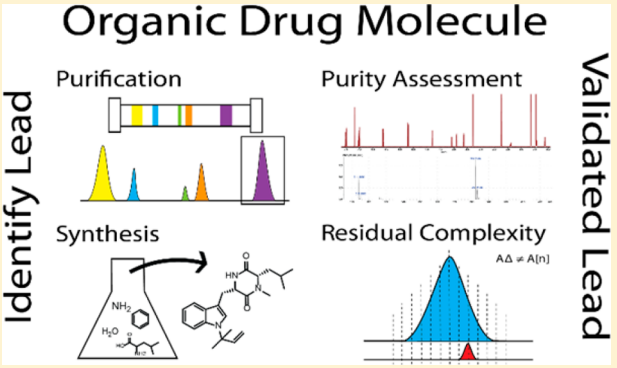
Supporting Information

ABSTRACT: Residual complexity (RC) involves the impact of subtle but critical structural and biological features on drug lead validation, including unexplained effects related to unidentified impurities. RC commonly plagues drug discovery efforts due to the inherent imperfections of chromatographic separation methods. The new diketopiperazine, rufomyazine (**6**), and the previously known antibiotic, rufomycin (**7**), represent a prototypical case of RC that (almost) resulted in the misassignment of biological activity. The case exemplifies that impurities well below the natural abundance of ^{13}C (1.1%) can be highly relevant and calls for advanced analytical characterization of drug leads with extended molar dynamic ranges of >1:1,000 using qNMR and LC-MS. Isolated from an actinomycete strain, **6** was originally found to be active against *Mycobacterium tuberculosis* with a minimum inhibitory concentration (MIC) of 2 μg/mL and high selectivity. As a part of lead validation, the dipeptide was synthesized and surprisingly found to be inactive. The initially observed activity was eventually attributed to a very minor contamination (0.24% [m/m]) with a highly active cyclic peptide (MIC ∼ 0.02 μM), subsequently identified as an analogue of **7**. This study illustrates the serious implications RC can exert on organic chemistry and drug discovery, and what efforts are vital to improve lead validation and efficiency, especially in NP-related drug discovery programs.



INTRODUCTION

Historically, chemists have turned to natural sources for novel scaffolds with appealing pharmacological activity. However, as many "low hanging fruit" have already been harvested, the quest has morphed into a search for minor active compounds or the development of methods for the expression of cryptic or silent biosynthetic gene clusters. This bears an increasing risk of false identification and/or misled assignment of active constituents. Both misidentification (real activity, but incorrect structural identity) and misassignment (activity assigned to the wrong component of a mixture) are common occurrences in natural product (NP) research and may be attributed to the inherent complexity of naturally derived chemical entities resulting from their metabolomic origin (see ref 1 and references therein). Low abundance constituents that remain throughout the process of fractionation may turn out to be active principles rather than the major, structurally assigned compound. This widespread experience raises the soul-searching question of how isolated an "isolated" NP actually is. In fact, perfect purity of final pharmaceutical products or active pharmaceutical ingredients (APIs) is difficult to achieve. Therefore, unless the composition of an API is thoroughly characterized, it is challenging to understand the effects that impurities or other structural variations, including conformers, may have on the biological activity. However, lead compounds in (NP) drug discovery programs are seldom characterized with the same scrutiny as pharmaceutical APIs intended for human use.

Incorrect chemical characterization leads to the disparity between resources spent on an early phase effort in contrast to the time spent on API validation. This may be due to an exponential relationship between resources spent and the progress achieved. The predominance of high-performance liquid chromatography (HPLC) for purity determination is mainly driven by resource considerations and exacerbates this disparity: representing a relative ratio method, HPLC by default produces outcomes that depend strongly on the chosen detector (specific vs universal) and evaluation model, including response

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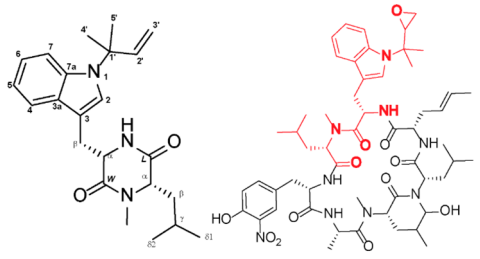
factors. In contrast, absolute methods allow definitive purity determination of the target analyte and provide more comprehensive information about the entirety of a sample. While absolute methods generally require greater resources, there is underutilized potential for even greater time and cost savings down the line when performed at early stages of development.

The extensive analysis performed on the taxol/paclitaxel pharmacophore illustrates both the difficulty that fully characterizing an API can entail and the importance of this effort: Snyder et al. were able to identify eight conformers by NMR when paclitaxel was studied in chloroform, some of them occupying ≤4% of the population.[2] Later, strong evidence was obtained to implicate the T-conformation as the preferred conformer bound to tubulin, and in vitro studies showed that this conformation is present at less than 5% of paclitaxel in solution.[3] The impact of such subtle, often structurally unexplained effects (static or dynamic) falls under the realm of residual complexity (RC; see also go.uic.edu/residualcomplexity).[4,5] RC refers to the "inherent deviation of nature-derived agents from single chemical entities caused by their metabolomic origin." However, more generally, RC refers to unexplained complexity within a sample, regardless of its origin (synthetic or natural), most commonly arising from the use of chromatographic purification. The paclitaxel case also serves as a reminder that highly active minor components, even in low abundance, can significantly affect the outcome of biological activity.

Interestingly, despite a general tendency to under report negative outcomes, the documented cases of misassigned activity can often be attributed to minor contamination arising from highly active components or misidentification of chemical structure. This study will use the term misassignment when referring to the false assignment of (or connection between) a specific chemical entity to an observed biological end point. In contrast, the term misidentification is used to designate errors in the process of structure elucidation or dereplication.

Recent work in our laboratory led to the isolation of rufomyazine (**6**; Chart 1), a new diketopiperazine, which after

Chart 1. Structures of Rufomyazine (6) and Rufomycin (7) with the Structural Similarities Highlighted in Red



a lengthy path of investigation eventually was recognized as another example of RC that led to the misassignment of activity to an inactive constituent. Originally isolated from the same extract as an analogue of the highly potent heptapeptide rufomycin (**7**),[6−9] **6** was evaluated with a minimum inhibitory concentration (MIC) of ∼5 μM against *Mycobacterium tuberculosis* (*M. tb*). Although not as highly potent as **7** (MIC ∼ 0.02 μM), the small molecular size and potential for less complex SAR studies made **6** appear as an interesting lead. Total synthesis and subsequent reisolation eventually led to the conclusion that **6** lacks inhibitory activity against *M. tb* completely. It is possible that **6** is a shunt metabolite off the biosynthetic pathway to **7**. Supporting this hypothesis, past work done by Schultz et al. has established that cyclomarazine, another dipeptide structurally similar to **6**, is a shunt metabolite of the biosynthetic pathway to cyclomarin, a heptapeptide structurally similar to **7**.[10] Therefore, a synthesis of **6** still acts as a stepping stone toward the further synthesis and development of **7**. The lack of antimycobacterial activity of the dipeptide also may contribute to the potential SARs of **7** as **6** represents roughly 1/3 of **7**'s structure. This suggests strongly that the required pharmacophore must contain more than just the Trp-Leu portion of **7**.

The isolation, structure elucidation, and total synthesis of **6** is presented below, in conjunction with a discussion of the global effects of RC in NP drug discovery and methodology to overcome this challenge.

RESULTS

Isolation and Structure Elucidation. Compound **6** was isolated from the *Streptomyces* strain, MJU3502, which was found to be 99.9% identical to *S. atratus* (NRRL B-16927) through classification using 16s rDNA sequence (Supporting Information). The isolation in 4 subsequent steps yielded a >98% pure white solid as determined by the 100% qNMR method.[11] The first step involved vacuum chromatography with MeOH/H_2O (see Experimental Section). The active fraction, VC4 (95% MeOH/H_2O), was selected for further fractionation via high speed countercurrent chromatography (HSCCC) using the solvent system HEMWat −1 (hexane: ethyl acetate: methanol: water, 6:4:5:5),[12] yielding nine recombined fractions as based on TLC monitoring. In step three, subfraction VC4-F4 was further fractionated by HSCCC using the orthogonal solvent system, HTerMWat −1 (hexane: methyl *tert*-butyl ether: methanol: water, 4:6:5:5); fractions were again recombined following analysis by TLC. Finally, fraction VC4-F4-C3 was subject to preparative HPLC to obtain compound **6**. Notably, although the 100% qNMR approach used for its purity assessment bears a certain risk of overestimating purity, just like any other nonabsolute quantitation, it still represents a highly practical purity assay. Importantly, 100% qNMR is universal due to its inherent capability of detecting both analogues as well as structurally unrelated but proton-containing impurities.

Structure elucidation was performed using a combination of HRMS and various 1D and 2D NMR techniques. Compound **6** was determined to be a cyclic dipeptide with a molecular formula of $C_{23}H_{31}N_3O_2$ and was recognized as a diketopiperazine by its general NMR characteristics. The monomeric units were identified as *N*-methyl-L-leucine (*N*Me-L-Leu) and 1-*N*-isoprenyl−L-tryptophan (prenyl−L-Trp). HRMS yielded a base positive ion of $[M + H]^+$ 382.2469 *m*/*z* and, after loss of the 1-*N*-isoprenyl group of Trp, an ion at $[M + H\text{-}68\ mu]^+$ 314.1830, indicating an exact mass of 381.24. The specific enantiomers come from ECD comparison with the synthetic material (see below). Compound **6** shared the same two sequential amino acids with **7**, suggesting that **6** may represent a shunt metabolite from the biosynthetic pathway leading to **7**.

Both the ^1H and the ^{13}C spectra (900 and 225 MHz, respectively) were well dispersed with minimal overlap, allowing for complete ^1H, ^{13}C, and HMBC assignments (Figure 1, Table 1). Initial examination of the ^1H NMR spectrum in CD_3OD confirmed the presence of 31 hydrogens, including one exchangeable proton. The exchangeable NH was observed at δ 8.206 ppm due to incomplete exchange. There was also a $^1H/^1H$−COSY correlation between the Hα of prenyl−L-Trp at δ 4.303 ppm and the exchangeable NH, confirming the position of the latter. The singlet at δ 2.788 ppm was indicative of an

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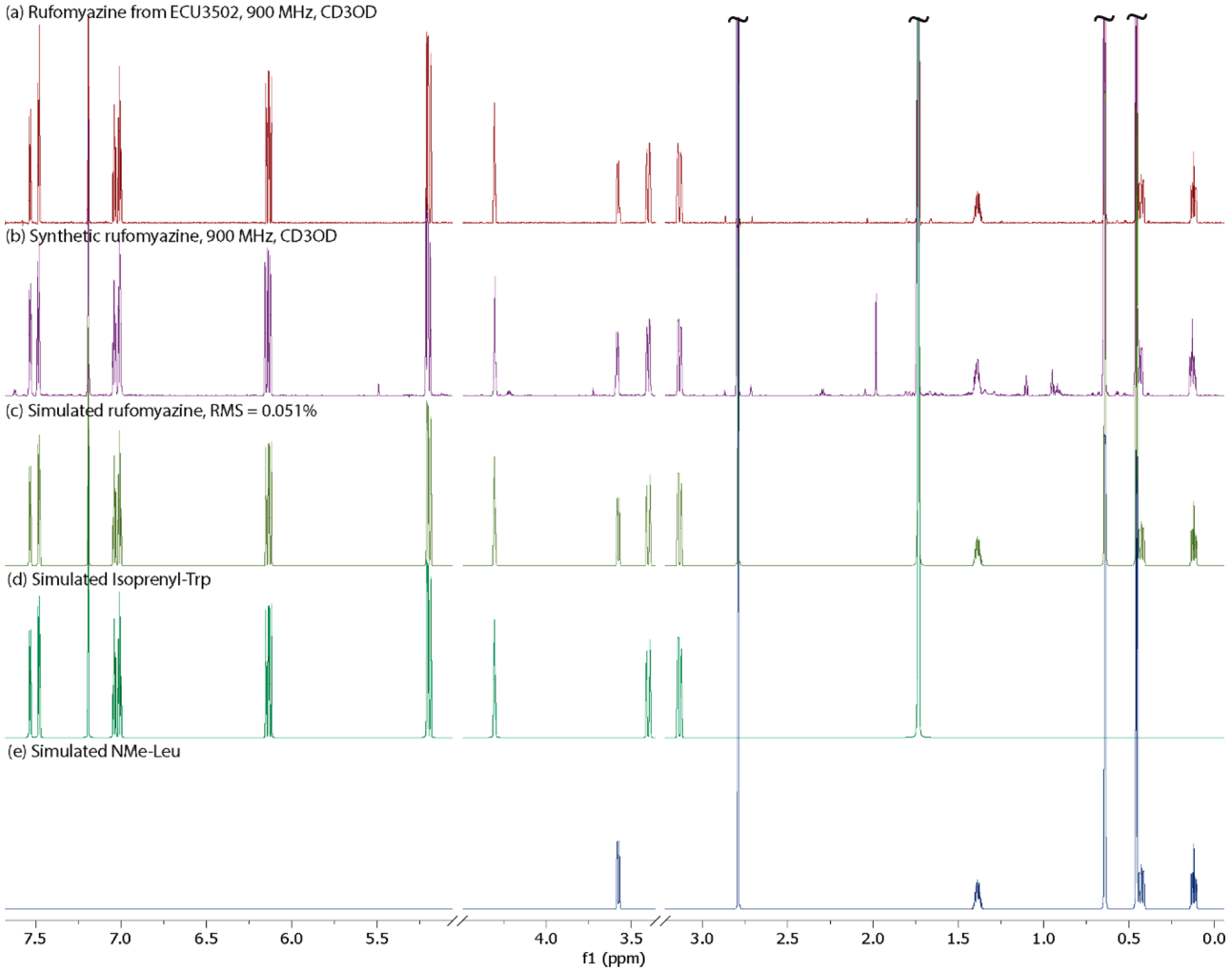

Figure 1. NMR spectrum of (a) isolated and (b) synthesized **6** (CD_3OD, 900 MHz). HiFSA simulated NMR profile of (c) isolated **6** and its constituent amino acids (d, e).

N-methyl group. Analysis of the ^{13}C-DEPT-Q-135 NMR spectrum in CD_3OD confirmed the presence of a total of 23 carbons reflected by 21 individual signals and a signal at δ 28.4 ppm representing two carbons. The signals at 169.7 and 168.3 ppm indicated the presence of two carbonyls, further indicating the dipeptidic structure of **6**.

The 5 ^{1}H NMR signals between 7.0 and 7.6 ppm confirmed the presence of a Trp residue with the characteristic fingerprint of an aromatic ABCD system, in addition to the pyrrole ring singlet (H-2) at δ 7.192 ppm. The tryptophan skeleton was confirmed by a COSY correlation sequence among the aromatic hydrogens H-4/5/6/7, as well as HMBC correlations from H-2 to C-3a, C-3, and C-7. The ^{1}H iterative full spin analysis (HiFSA) of prenyl−L-Trp (Figure 1) indicated the presence of two nearly equally abundant, distinct rotamers (ratio: 0.55:0.45). The presence of rotamers became most obvious from the signals of the Hβ methylene hydrogens of Trp at δ 3.402 and 3.133 ppm, where processing with Lorentzian−Gaussian window functions reveals additional splitting of the *dd* signals correlating to the two distinct rotamers. Connectivity to the carbonyl was confirmed through an HMBC correlation of the Hβ methylene pair to the Trp carbonyl at δ168.3 ppm. Attachment of the isoprenyl group to N-1 was confirmed through HMBC correlation between H-2 and C-1′. In addition, the signals at δ 6.134, 5.205, and 5.194 ppm were indicative of a terminal double bond. The *J* coupling pattern of H-2′, H-3a′, and H-3b′ (Table 1) clearly indicated a terminal double bond, confirming the isoprenyl partial structure.

The five signals between 0.4 and 1.4 ppm confirmed the presence of a Leu residue. The highly coupled *ddqq* signal at δ 1.386 ppm, in combination with the two *d* methyl signals at δ 0.641 and 0.454 ppm, were characteristic of Leu. Additionally, the COSY spectrum clearly connected the entire aliphatic chain of Leu as a continuous spin system. Connectivity of the *N*-methyl was confirmed by the HMBC correlation between Hα of *N*Me-L-Leu and the *N*Me carbon. The HMBC spectrum also confirmed connectivity to the carbonyl at δ 169.7 ppm via a correlation to the Hβ. The lack of observable HMBC correlations from the γ proton is best explained by its dihedral angles with ^3J carbons being near 90°. Finally, the cyclic nature of **6** was confirmed through Hα and carbonyl HMBC correlations connecting Hα-Leu with C═O-Trp and Hα-Trp with C═O-Leu.

Total Synthesis. Scheme 1 summarizes the total synthesis of **6**. The intermediate **2** was prepared following the work published by Luzung et al. with yields that ranged from 51 to 69%.[13] The sequence was followed from the literature[14−17] for related compounds and was found to proceed readily to the product (see Experimental Section).

Electronic circular dichroism (ECD) analysis of synthetic **6** containing only L-amino acids showed a positive Cotton effect at 234 nm and a negative Cotton effect at 220 nm. Natural **6**

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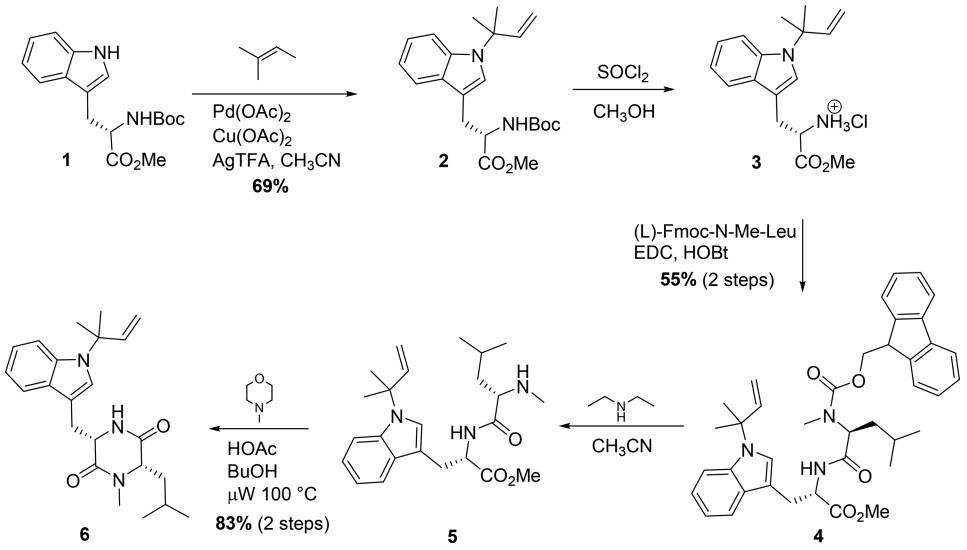
Table 1. 900 MHz NMR Data of 6 (in CD_3OD; δ_H and J Determined by HiFSA)

unit	position	δ_C, type	δ_H (J in Hz)	$^1J_{CH}$ (Hz)[a]	HMBC[b]
prenyl–	C=O	168.32, C			
L-Trp	α	57.93, CH	4.303, dd (4.47, 4.08)	142.6	β, 3, C=O^W, C=O^L
	β	30.97, CH_2	3.402, dd (−14.8, 4.08)	131.0	α, 2, 3, 3a, C=O^W
			3.133, dd (−14.8, 4.47)	131.7	
	2	126.45, CH	7.192, s	181.0	β, 1′, 3, 3a, 7a
	3	108.61, C			
	3a	131.30, C			
	4	120.34, CH	7.533 ddd (7.98, 1.21, 0.75)	158.1	3, 6, 7a
	5	120.14, CH	7.008, ddd (7.98, 6.91, 0.88)	153.8	3a, 7
	6	121.80, CH	7.039, ddd (8.40, 6.91, 1.21)	158.2	4, 7a
	7	114.94, CH	7.482, ddd (8.40, 0.88, 0.75)	160.0	5, 3a
	7a	136.90, C			
	1′	60.12, C			
	2′	145.60 (CH)	6.134, dd (17.5, 10.7)	156.4	1′, 4′, 5′
	3′	113.95, CH_2	5.205, dd (10.7, −0.84)	159.3	1′, 2′, 4′, 5′
			5.194, dd (17.5, −0.84)	154.7	
	4′	28.40, CH_3	1.730, s	127.8	1′, 2′, 3′, 5′
	5′	28.40, CH_3	1.736, s	127.8	1′, 2′, 3′, 4′
	NH		8.206, s[b]		
*N*Me	C=O	169.66, C			
L-Leu	α	60.84, CH	3.578, dd (8.71, 4.02)	143.0	γ, β, C=O^W, C=O^L
	β	43.28, CH_2	0.426, ddd (−14.1, 8.83, 4.02)		γ, α, L$^{C=O}$
			0.119, ddd (−14.1, 8.71, 5.53)		
	γ	26.55, CH	1.386, ddqq (8.83, 6.68, 6.54, 5.53)		
	δ1	21.97, CH_3	0.641, d (6.54)	123.8	β, γ, δ2
	δ2	23.23, CH_3	0.454, d (6.68)	124.7	β, γ, δ1
	NMe	33.33, CH_3	2.788, s	139.3	α, C=O^W

[a]Measured from ^{13}C satellites in the ^1H NMR spectrum. [b]Stated from hydrogen to indicated carbon. [c]Observed due to incomplete exchange.

Scheme 1. Total Synthesis of 6



showed identical Cotton effects, indicating that both compounds have the same absolute configuration.

Residual Complexity Explains Misassigned Bioactivity. Upon initial isolation of **6**, structure elucidation (via NMR and HRMS) and biological activity assays were run in parallel due to the required 7-day anti-*M. tb* bioactivity assay. When ^1H qNMR (qHNMR, 100% method) and HRMS indicated a >98% purity of the sample used for structure elucidation, there was no evidence

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of, and thus minimal concern for, chemical contamination interfering with biological evaluation. Therefore, when the relatively small diketopiperazine molecule showed an average MIC of ~5 μM (2.0 ± 0.14 μg/mL) against *M. tb*, synthesis seemed to be a natural next step to facilitate SAR studies. However, the synthetic product, though structurally identical with natural **6**, failed to show any biological activity against *M. tb*. This led to exploratory measures to determine the source of the activity present in the isolated material.

The remaining stock from the sample used for biological testing (~1 mg) was recovered, lyophilized, and dissolved in CD_3OD for NMR analysis. The 900 MHz data (S/N [5.20 ppm] = 168) did not indicate any change in chemical composition except for the presence of residual solvent. However, as the sample was less than 1 mg and not crystalline, the potential for minor contamination from a highly potent compound such as **7** had to be considered. The NMR sample was again recovered, diluted with LCMC grade MeOH, and analyzed via HPLC-MS in scanning mode in an attempt to identify the source of bioactivity through the presence of an impurity. The results confirmed the presence of trace amounts primarily **6** and **7**. Based on the MIC values of **7** (0.02 ± 0.03 μg/mL) and the MIC (~2 μg/mL) of the contaminated sample of **6**, it was estimated that the sample contains ~1% or less of **7**. For more precise quantification of the bioactive, contaminated sample of **6**, an MRM-HPLC-MS method was established that used calibration with **7**. Thereby, the sample was shown to contain 0.24% of **7**, which is equivalent to a ~1:400 (w/w) ratio of **6** and **7**. Factoring in the 3:1 MW ratio between **7** and **6**, this translates into a ~1:1,200 dynamic range for the purity analysis at hand. This explains why the larger cyclic peptide **7** was not seen in the initial NMR spectra, including the first qHNMR analysis: ignoring the impact of signal multiplicity, the resonances of the bioactive **7** were ~6-fold less intense than the ^{13}C satellite signals (0.55%, equivalent to a 1:200 dynamic range) of the major compound, **6**.

To visualize the impact of such a strikingly low degree of biologically relevant impurity on the drug discovery workflow, it can be estimated that ~10 mg of sample would be needed, acquiring 256 scans on a 600 MHz NMR instrument equipped with a 5 mm cryoprobe to capture the observed 1:1,200 dynamic range. As this is well beyond what typical purity evaluations cover, it raises the broader question about minimum requirements for the RC assessment of biologically active lead samples.

The initial estimate of ~1% contamination, based on biological activity, was off by 4-fold, a difference which commonly and reasonably is attributed to the typical standard deviation of bioassays. However, it is also important to consider purity adjusted bioactivity for any control substances used: for the present rufomycin standard, the purity adjusted MIC was 0.013 μg/mL, thus lowering the estimated rufomycin impurity content from ~1.0 to ~0.6%, which is only 3-fold different from the mass-based analysis. To perform the purity-adjustment with confidence, the purities of synthetic rufomyazine and the rufomycin standard were assessed by two qNMR (absolute and relative) and two UHPLC methods (Table 2). The expected lower values revealed by the absolute methods could be explained by the presence of inert components such as solvation water (33%, equivalent to 20 molecules of bound water) or proton-free impurities secondary to the isolation process by preparative solid phase HPLC.

In addition, to rule out potential for synergistic effects between **6** and **7**, synthetic **6** was assessed biologically in a checkerboard format with **7**. The average FIC index value obtained was

Table 2. Comparison of the Purity Values (% w/w) of Synthetic Rufomyazine (6) and Rufomyazine (7) Reference Standard (Including Breakdown into Conformers) As Determined by qNMR (Absolute qNMR with 3,5-Dinitrobenzoic Acid as Internal Calibrant (IC) and 100% Method) and UHPLC

	abs-qNMR IC	qNMR-100	UHPLC (method 1)	UHPLC (method 2)
synthetic rufomyazine (6)	**85.45**	**97.45**	**94.82**	**93.35**
rufomycin (7)	**66.99**	**94.1**	**95.47**	**94.88**
conformer 7-1	50.33	70.55		
conformer 7-2	7.82	11.06		
conformer 7-3	8.84	12.49		

0.38−1.09, which might indicate synergistic or additive effects; however, this was nonconclusive and difficult to determine accurately due to the lack of activity of **6**. The large range of FIC led to a no-effect conclusion.

The source of the impurity does not affect the analytical challenge but shall be addressed briefly despite the lack of conclusive evidence. As **6** and **7** were isolated from the same actinomycete strain, same mother fraction, and baseline separated in the same chromatogram (see Supporting Information), contamination during sample handling or isolation cannot be ruled out. An alternative explanation relates to sequential injections during semipreparative chromatography, suggesting that there may have been residual **7** carry-over between injections. This hypothesis was given some preference as **7** analogues tend to elute toward the end of the chromatogram, while **6** elutes early. It is possible that carry-over of **7** into the collection of **6** was possible. The contamination of **6** by **7** was not anticipated due to drastic difference in retention time and the observations in the NMR spectra.

DISCUSSION

Lessons Learned and Implications for NP-Based Drug Discovery. The isolation and discovery of dipeptide **6** via bioactivity-guided fractionation has illuminated potential means for the improvement in (NP) drug discovery workflows. It is therefore important to consider the causality behind known instances of RC and methods that can be employed to avoid these diversions. Considering the metabolomic complexity of NPs, which are sourced from crude extracts and fractions, chromatographic methods are inevitable for the identification of the active components. Preparative HPLC represents the mainstay chromatography in both academic and industrial settings but contains several imperfections that are well understood and can result in the impact of RC on the overall outcome. Countercurrent separation (CCS) may offer an alternative solution with a reduced risk of carryover from run-to-run and an intrinsic lack of column memory effects, as the column is a liquid (stationary phase) that is typically extruded and/or replaced.[18]

Additional methods exists to assist in the identification of underlying minor active components: for example, the establishment of purity activity relationships (PARs),[4] which correlate the purity of an isolate with its bioactivity, and its fully quantitative variant, qPAR,[19] can uncover a misassignment as a miscorrelation between detectable chemical species and observed biological end points.

A conceptually orthogonal approach to purity and (q)PAR evaluations is confirmatory synthesis. As exemplified here, it represents a vital element of lead validation, provided the isolated

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compound lends itself to synthesis. In practice, this frequently represents a limitation faced by the NP researchers who isolate the compound and/or perform biological studies. As discussed by Nicolaou and Snyder,[20] even with modern techniques such as MS, NMR, and X-ray crystallography, structure elucidation of NPs is not a trivial task. Their review mentions several examples in which total synthesis played a crucial role in the identification of the originally isolated NP. In fact, structure revision relied on total synthesis in many of these cases. The present study re-emphasizes the relevance of synthesis for lead validation for an RC case of a low-abundance, high-activity impurity. Synthesis may serve to confirm or refute biological activity that was initially observed for the compound. It should be kept in mind that synthesis can also lead to its own RC due to synthetic byproducts, especially but not limited to circumstances where intermediates are not thoroughly purified. While likely applicable to a lesser degree than metabolomic NPs, RC of synthetic compounds should always be considered as a potential factor of observed bioactivity.

General Conclusions. In light of the above, a general conclusion is that the purity of synthetically produced compounds and materials isolated from a natural source can be critically important, and that purity determination should always be coupled with structural validation.[11] A small contamination with a highly potent compound can lead to a drastic increase in bioactivity, as was the case with **6** contaminated with **7**. Due to the high potency of the heptapeptide, **7**, a contamination level as low as 1:400 (w/w), equivalent to 1:1,200 (mol/mol), was sufficient to yield a deceptively potent MIC for the purified "lead" compound. This indicates that the more potent the contaminant, the more sensitive and exhaustive the associated purity analysis must be.

Of the four most used methods for purity assessment, (U)HPLC, TLC, HPLC-MS, and NMR, the first two are the by far most frequently used, but generally the least specific and least sensitive at the same time. Moreover, these methods represent relative-selective methods, as represented in Figure 2,

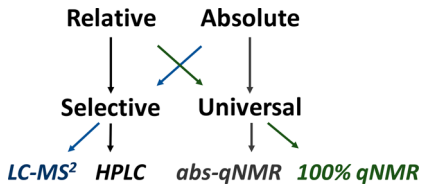

Figure 2. Categorical representation of four approaches to quantitation in purity determination, generated by the two pairs of principal quantitation (relative vs absolute) and detection (selective vs universal) modes: (a) HPLC-MS^2: absolute-selective; (b) HPLC: relative-selective; (c) absolute qNMR (abs-qNMR): absolute-universal; (d) 100% qNMR: relative universal.

which shows the four principal types of quantitation. A relative-selective method measures purity for only the desired compounds and may under-represent or completely overlook any other constituents due to overlap in retention time, UV transparency, or any other form of remaining undetected. In the present study, the low-abundance but highly active impurity, **7**, was eventually identified as a chemical entity that was known from the same crude NP. This knowledge ultimately enabled the ability to take advantage of the sensitivity of HPLC-MS for the identification of the minor bioactive. In contrast to (U)HPLC, HPLC-MS, and TLC, HPLC-MS^2 (tandem MS) represents an absolute-selective method: it can determine absolute purity when combined with standard calibration curve. However, this is only feasible for known compounds that are ionizable and for which identical reference standards are available. Unknown components cannot be quantitated, and failure of analytes to ionize will inevitably make them go unnoticed.

The unexpectedly large dynamic range of the RC issue at hand (1:1,200) precluded the feasibility of a qHNMR analysis for the given 1 mg sample. However, absolute qNMR (abs-qNMR) represents an absolute-universal purity determination method (Figure 2) capable of assessing the absolute purity of a sample, including residual solvent or other bound components, and works for any molecule containing hydrogen. In comparison, 100% qNMR is a relative-universal method. Unlike abs-qNMR, it cannot quantitate residual water. While, much like MS, 100% qNMR must know the identity of the quantified species in a mixture, it has the advantage of typically providing valuable structural information via the observed impurity signals.

Collectively, compound **6** represents an exemplary case of how RC can affect drug discovery efforts. The downstream effects are substantial, as time and effort placed on a potential lead grows exponentially as the lead progresses through further development. Despite the apparent lack of reporting on cases of misassignment and misidentification, there have been several cases reported that can serve as a warning to all chemists. A striking example for this broader impact is the case of diazonamide A,[21−23] as its original assigned structure was incorrect due to misinterpretation of the X-ray crystallography data. This led to years of effort chasing a structure that never existed.

An additional example of misidentification was found by Lear et al. and involved the synthesis of lassomycin, a cyclic peptide originally found to target ClpC1 and to be highly active against *M. tb*.[24] Following synthesis of lassomycin and lassomycin-amide, neither of the compounds were found to be active against *M. tb*. The authors hypothesized that lassomycin may exhibit a threaded conformation rather than the unthreaded structure that was synthesized from the originally reported structure. Therefore, structural conformation of lassomycin may have been misidentified. A recent report of RC by Grzelak et al. may serve as a final example: two isoflavones that were isolated from an actinomycete extract showed >90% purity and MICs of 0.75 and 0.11 μg/mL against *M. tb*.[25] However, synthesized material of these compounds failed to demonstrate any anti-*M. tb* activity. TLC-bioautography in combination with MS and NMR confirmed that the isoflavone samples contained a potent cyclic heptapeptide, xylamycin, which had coeluted with the isoflavones in the initial isolation (manuscript in preparation).

A case of misassigned activity arising from minor bioactives was uncovered by the synthesis of epiquinamide performed by Fitch et al.[26] Upon completion of the synthesis, the authors found epiquinamide to be inactive at nicotinic receptors even though observed activity was detected from what they thought was the isolated species. Consecutively, the activity was attributed to possible contamination with epibatidine, a potent agonist. A similar case reported by Pettit et al. involved the laborious synthesis of dolastatin-16, a cyclodepsipeptide.[27] Originally, dolastatin-16 was isolated from *Dolabella auricularia* and found to be a potent inhibitor of cancer cell growth; in fact, one of the most potent of such agents reported from the Pettit group over four decades of the group's drug discovery enterprise.[28] As in the epiquinamide case, the synthetic dolastatin-16 showed no inhibitory effects. Despite major efforts, the authors have been unable to successfully link the previously reported biological activity to a definitive structural entity. The loss of activity

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was attributed to either an unidentifiable contaminat or a solvent-induced conformational change associated with the synthetic compound. On a more encouraging note, the Matsuda group synthesized dolastatin-16 and found it to be a potent antifouling agent against *Amphibalanus amphitrite*,[29] suggesting that a lack of cancer cell inhibition may not be the end of the development prospects of dolastatin-16.

Each of these examples represents a case where (chemical) misidentification or (chemical/biological) misassignment occurred, most likely attributable to RC. Cases such as these and the new case presented here can be avoided or minimized through increased diligence of lead validation by early purity analysis through appropriate methods, confirmatory chemical synthesis, and awareness of RC phenomena that can break the linkage between chemical structure and biological activity.

EXPERIMENTAL SECTION

General Experimental Procedures. All reagents were purchased from Sigma-Aldrich Chemical Co. or Fisher, unless otherwise specified. Amino acids and protected amino acids were purchased from Chem Impex and used without further purification. Vacuum liquid chromatography was carried out using Polygoprep 100−50C_{18} (Macherey-Nagel) with 5 subsequent single column volumes of MeOH/H_2O (20, 60, 85, and 95%) and 100% $CHCl_3$. Each fraction was collected separately. HSCCC was carried out using a 320 mL Pharma Tech HSCCC (model CCC-1000, PharmaTech Research Corp.) instrument in descending mode with a flow rate of 1.5 mL/min and a rotational speed of 900 rpm. The solvent systems used were HEMWat −1 at a ratio of 6:4:5:5 v/v and an orthogonal system of HTerMWat +1 at a ratio of 4:6:5:5 v/v. Both solvent systems were chosen on the basis of the GUESS method.[12,30] Preparative reverse phase HPLC was performed using a Waters Delta 600 (Waters Corp.) instrument and a YMC-Pack ODS-AQ C18 semipreparative column (250 × 10 mm ID, 5 μm particle size, 12 nm pore size, YMC). The column was eluted with a H_2O/ACN gradient containing 15% ethyl acetate and 0.1% FA at a flow rate of 3 mL/min. The gradient was 50 to 70% for 21 min, isocratic for 4 min at 70%, 70 to 50% for 3 min, and isocratic at 50% for 8 min. Purity assessment by UHPLC was performed using Shimadzu HFLC (Shimadzu Corp.) Nexera UHPLC system equipped with a DAD detector. An Acquity UPLC BEH C18 column (2.1 × 50 mm ID, 1.7 μm particle size, Waters Corp.) was used for separation. Analysis was carried out using the Shimadzu Lab Solutions software package. Specific detail on the gradients used can be found in the Supporting Information. Synthetic chromatographic purifications were performed using prepacked FLASH silica cartridges from Biotage or an HPFC Biotage SP1 system using prefilled KP-Sil (normal phase) SNAP cartridges. UV detection at 264 and 245 nm was used to monitor progress utilizing a hexane/ethyl acetate gradient. For sealed microwave heated reactions, a CEM Explorer 48/72/96 automated microwave synthesizer utilizing a floor mounted IR temperature sensor controlled by an external computer loaded with Synergy application software (Version 1.1) was used.

ECD spectra were measured on a Jasco-815 spectropolarimeter using methanol with a 0.2 cm path length cuvette at 25 °C. The ^{1}H and ^{13}C NMR spectra were recorded at 900 and 225 MHz, respectively, on a Bruker Avance 900 NMR spectrometer (Bruker BioSpin) equipped with an AVANCE II console. The spectrometer was equipped with a 5 mm TCI triple resonance inverse detection cryoprobe with a *z*-axis pulse field gradient. The ^{1}H spectra collected during synthesis were recorded on Bruker DPX spectrometer at 400 MHz. The absolute qNMR experiments were carried out on a Bruker Avance 800 NMR instrument (Bruker BioSpin) equipped with a 5 mm TXI probe. Specific details about absolute qNMR experiments including processing can be found in the Supporting Information. Coupling constants are expressed in Hertz, and chemical shifts are reported in ppm with reference to the residual protonated solvent MeOH at 3.310 ppm, DMSO at 2.500 ppm, and $CHCl_3$ at 7.260 ppm for ^{1}H. For ^{13}C, reference to residual MeOH at 49.00 ppm, DMSO at 39.52, and $CHCl_3$ at 77.16 ppm was used. Computer assisted ^{1}H iterative full spin analysis (HiFSA) was performed using PERCH NMR Tools (v.2014.1 and v.2015.1, PERCH Solution Ltd.). High resolution HPLC-MS were obtained with a Shimadzu LCMS-ion trap (IT)-time-of-flight (TOF) mass spectrometer with electrospray ion source. Analysis of the contaminated biological sample was performed with an AB MDS Sciex 4000 quadrupole linear trap (Q Trap) spectrometer with electrospray ion source.

***N*-(1,1-Dimethyl-1-ally)-L-Trp-Ome Hydrochloride (3).** Thionyl chloride (47 uL, 0.64 mmol, 1.5 equiv) was added to ice-cooled methanol (5 mL) while stirring. A solution of intermediate *N*-(1,1-dimethyl-1-allyl)-L-Boc-Trp-OMe[13] (**2**) (166 mg, 0.43 mmol) in MeOH (5 mL) was added to the chilled solution. The mixture was immersed in a preheated oil bath (50 °C) for 2 h at which time TLC analysis (20% EtOAc:hexanes) shows no starting material remaining. The solvents were evaporated from the reaction mixture to give 0.142 g (crude yield 98%) of the product **3** as an oil. This unpurified material was used directly for the next step.

Fmoc-L-*N*-Me-Leu-*N*-(1,1-dimethyl-1-allyl)-L-Trp-OMe (4). To a 0 °C solution of Fmoc-L-*N*-Me-Leu-OH (0.16 g, 0.43 mmol, 1 equiv) in DCM (10 mL) were added successively HOBt (64 mg, 0.47 mmol, 1.1 equiv), EDCI (91 mg, 0.47 mmol, 1.1 equiv), and DIPEA (85 uL, 0.475 mmol, 1.1 equiv). The mixture was stirred for 20 min under nitrogen at which time a solution of DIPEA (85 uL, 0.475 mmol, 1.1 equiv) and *N*-(1,1-dimethyl-1-ally)-L-Trp-OMe hydrochloride (**3**) (0.142 g from above) in DCM (10 mL) was added, and this mixture was stirred at RT until complete by TLC analysis (50% EtOAc:hexanes). The reaction was quenched with 5% $KHSO_4$ solution at 0 °C and extracted with DCM (3 × 10 mL). The combined organic layer was washed with brine and dried over Na_2SO_4, and the solvents were evaporated to give crude foam: yield 0.289 g. The crude material was purified by silica gel chromatography via Biotage SP1 using 25% EtOAc/Hex to give 0.150 g foam (55% yield for two steps). ^{1}H NMR (CD_3OD, 400 MHz) δ 7.76 (2H, m), 7.55 (2H, m), 7.45 (1H, m), 7.40 (1H, m), 7.36 (2H, m), 7.28 (2H, m), 7.16 (1H, s), 6.99 (1H, m), 6.96 (1H, m), 6.07 (1H, dd, *J* = 17.4, 10.7 Hz), 5.12 (1H, dd, *J* = 10.7, 0.8 Hz), 5.04 (1H, dd, *J* = 17.4, 0.8 Hz), 4.68 (1H, dd, *J* = 8.8, 5.1 Hz), 4.66 (1H, m), 4.56 (1H, dd, *J* = 10.2, 6.0 Hz), 4.33 (1H, dd, *J* = 10.2, 6.2 Hz), 4.18 (1H, dd, 6.0, 5.2 Hz), 3.69 (3H, s), 3.29 (1H, dd, *J* = 14.6, 5.1 Hz), 3.14 (1H, dd, 14.6, 8.8 Hz), 2.48 (3H, s), 1.66 (6H, s), 1.50 (1H, m), 1.40 (1H, m), 1.25 (1H, m), 0.88 (3H, d, 6.6 Hz), 0.84 (3H, d, 6.4 Hz). ^{13}C NMR (CD_3OD, 400 MHz) δ 173.7, 173.4, 158.4, 145.5, 145.3, 142.6 (2C), 136.9, 130.6, 128.8 (2C), 128.8 (2C), 128.1 (2C), 125.8 (2C), 125.1, 121.7, 120.9 (2C), 119.9, 119.4, 115.0, 113.9, 109.5, 68.52, 57.9, 54.5, 52.8, 48.3, 38.3, 30.1, 28.4, 28.3, 27.9, 25.8, 23.5, 22.0. HRMS (ESI) *m*/*z* 636.3422 $[M + H]^+$ (calculated for $C_{39}H_{45}N_3O_5$, 635.3359). Purity 93% determined by abs-qNMR-IC.

H-L-*N*-Me-Leu-*N*-(1,1-dimethyl-1-allyl)-L-Trp-OMe (5). Compound **4** (274 mg, 0.43 mmol) was dissolved in acetonitrile (4 mL) and treated with diethylamine (4 mL) at RT with stirring. This mixture was stirred for 1 h at which time TLC analysis (20% EtOAc:hexanes) showed no starting material. The solvents were evaporated, and the crude residue was used directly for next step.

Rufomyazine (6) Synthesis. Crude **5** (from 0.43 mmol **4**) was placed in a 10 mL microwave vessel along with acetic acid (0.37 mL, 6.5 mmol, 15 equiv) and *N*-methylmorpholine (0.26 mL, 2.4 mmol, 5.5 equiv). The mixture was heated to 100 °C in microwave for 15 min, at which time TLC analysis (3% MeOH/DCM) showed no starting material. The solvents were evaporated; the crude residue was triturated in methanol and filtered, and the resultant filtrate was dried in vacuo to obtain 40.2 mg of white crystalline solid (83% yield for two steps). ^{1}H NMR (CD_3OD, 900 MHz) δ 7.53 (1H, ddd *J* = 7.8, 1.3, 0.7 Hz), 7.48 (1H, dt, *J* = 8.3, 0.9 Hz), 7.19 (1H, s), 7.04 (1H, ddd, *J* = 8.3, 6.9, 1.3 Hz), 7.01 (1H, ddd, *J* = 7.9, 6.9, 1.0 Hz), 6.13 (1H, dd, *J* = 17.5, 10.5 Hz), 5.21 (1H, dd, *J* = 10.7, 0.9 Hz), 5.20 (1H, dd, *J* = 17.5, 0.9 Hz), 4.30 (1H, t, *J* = 4.2 Hz), 3.58 (1H, dd, *J* = 8.8, 4.0 Hz), 3.40 (1H, dd, *J* = 14.8, 4.1 Hz), 3.13 (1H, dd, *J* = 14.8, 4.5 Hz), 2.79 (3H, s), 1.73 (3H, s), 1.74 (3H, s), 1.39 (1H, ddqq, *J* = 9.1, 6.7, 6.5, 5.5), 0.64 (3H, d, 6.5 Hz), 0.46 (3H, d, 6.7 Hz), 0.44 (1H, ddd, *J* = 14.2, 9.1, 4.2 Hz), 0.13 (1H, ddd, *J* = 14.2, 8.7, 5.5 Hz). ^{13}C NMR (CD_3OD, 900 MHz) δ 169.7, 168.3, 145.6, 136.9, 131.3, 126.4, 121.6, 120.3, 120.1, 114.9, 113.9, 108.6, 60.8, 60.13, 57.9,

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43.3, 33.3, 31.0, 28.4, 26.6, 23.2, 21.9, HRMS (ESI) m/z 382.2469 $[M + H]^+$ (calculated for $C_{23}H_{32}N_3O_2$, 382.2494).

***M. tb* Assay Procedures.** MIC was assessed using *M. tb* strain $H_{37}Rv$ (ATCC 27294). All MICs were determined using the Microplate Alamar Blue Assay (MABA).[31] The MIC was defined as the lowest concentration of compound to exhibit a 90% reduction in fluorescence relative to untreated controls.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.8b00988. The raw NMR data (FIDs) are available at DOI: 10.7910/DVN/YHUB8D.

Copies of the ^1H, ^{13}C, COSY, HSQC, HMBC, and HiFSA profiles of **6** and **4**; ^1H, ^{13}C, and COSY spectra of the synthetically produced compound **6**; HRMS spectrum of **6** and **4**; MS calibration curve for the LC-MS quantitation of **7** with the NMR analysis of the contaminated sample (PDF)

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Notes

The authors declare no competing financial interest.

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