Structure Elucidation

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Reassignment of the Structure of the Antibiotic A53868 Reveals an Unusual Amino Dehydrophosphonic Acid**

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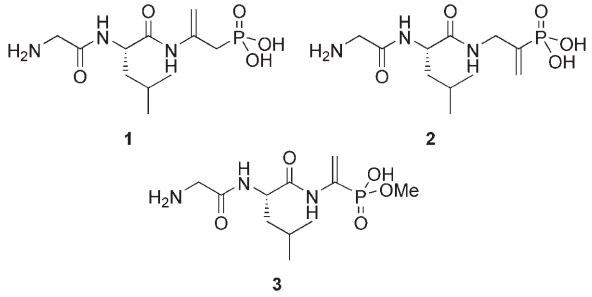
Antibiotic biosynthesis has enjoyed a renewed interest in recent years, with most efforts directed at polyketide synthases, non-ribosomal peptide synthetases, and antimicrobial peptides.[1,2] Relatively unexplored with respect to biosynthetic pathways are phosphonate antibiotics, despite their commercial use as pharmaceuticals, herbicides, and pesticides.[3–5] In fact, currently the biosynthetic pathways of only three phosphonate antibiotics are known: fosfomycin,[6] bialaphos,[7] and FR900098.[8] These studies and others[9] have uncovered intriguing new biochemical transformations involved in phosphonate biosynthesis. As part of a program to provide more insights into the biogenesis of this class of compounds, we investigated A53868, the structure of which was originally reported as **1**[10] but later revised to **2** (Scheme 1).[11] We show here that the actual structure of the compound is, however, the unusual amino dehydrophosphonic acid **3**.



Scheme 1. Previously reported (**1**) and reassigned (**2**) structure of A53868, along with the structure assigned in this work (**3**).

A53868 is produced by *Streptomyces luridus* and has broad spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria.[10] Analysis of the spent medium of the producing strain shows a characteristic signal for a phosphonate with a chemical shift around $\delta = 10$ ppm in the ^{31}P NMR spectrum (Figure 1 a). Synthesis of the reported structure **2** was achieved by Ni-catalyzed addition[12] of dibenzylphosphite to the protected propargylamine **4** to

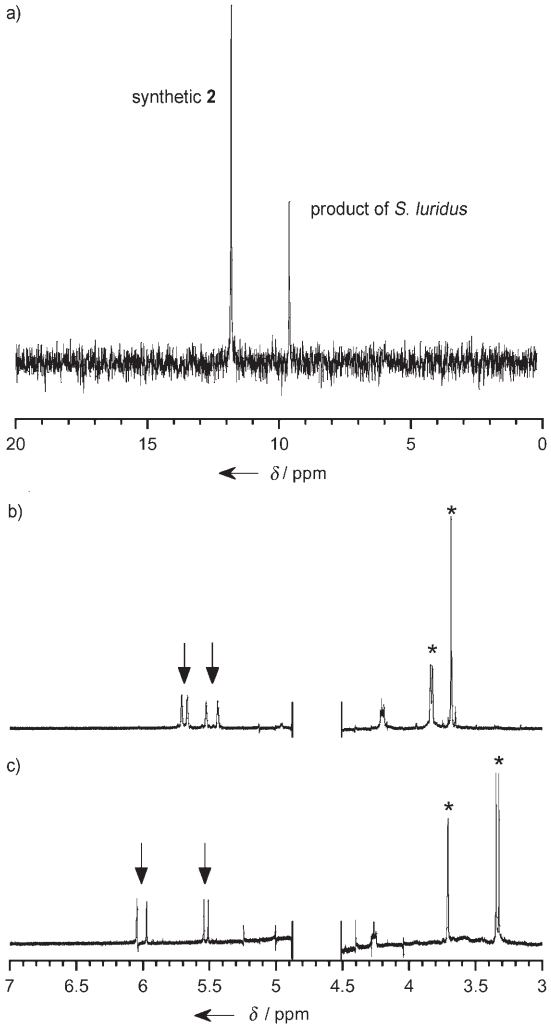


Figure 1. a) ^{31}P NMR spectrum of A53868 isolated from *S. luridus* and synthetic **2**. b) ^{1}H NMR spectrum of synthetic peptide **2**. c) ^{1}H NMR spectrum of A53868 from *S. luridus*. The spectra were recorded in D_2O, and the water peak is omitted in (b) and (c) for clarity. The arrows and asterisks are explained in the text.

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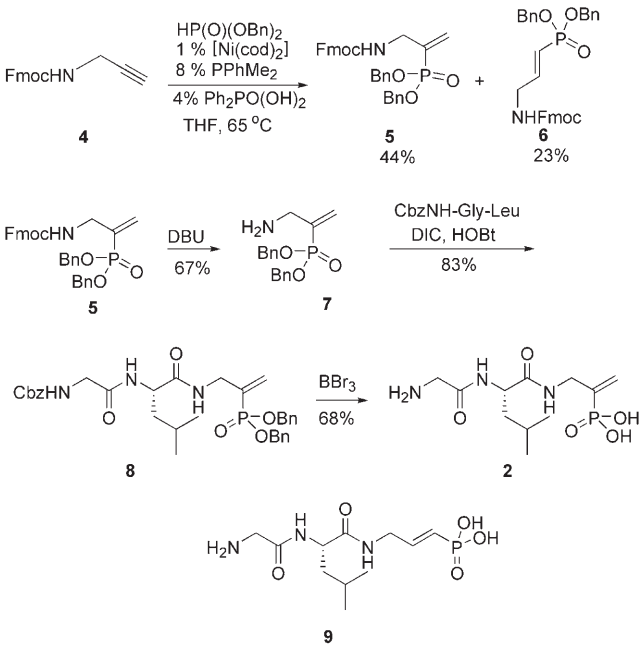
afford vinylphosphonate **5** and its constitutional isomer **6** (Scheme 2). Both compounds were separated, deprotected, and coupled to the protected dipeptide CbzNH-Gly-Leu by

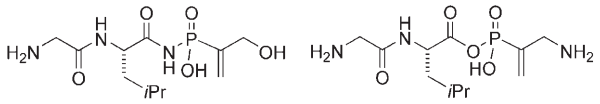


Scheme 2. Synthetic route to peptide **2** and isomer **9**. Fmoc = 9-fluorenylmethoxycarbonyl, cod = 1,5-cyclooctadiene, Bn = benzyl, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, DIC = 1,3-diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole, Cbz = benzyloxycarbonyl.

solution-phase peptide synthesis. After global deprotection with boron tribromide,[13] peptide **2** and its isomer **9** were obtained.

Surprisingly, both synthetic phosphonates displayed different ^{31}P NMR spectra than the material produced by *S. luridus* (see Figure 1a for a mixture of the natural product and synthetic **2**). The natural product was therefore purified from the spent medium of *S. luridus* by HPLC, and its ^{1}H NMR spectra was recorded (Figure 1c), again showing different resonances compared to the NMR spectra of the synthetic compounds **2** (Figure 1b) and **9**. Whereas a *trans*-vinylphosphonate structure such as in **9** can be readily ruled out for A53868 from the NMR data, the spectra of the natural product and **2** are remarkably similar, both exhibiting two vinyl protons with clear splittings owing to interaction with the phosphorus atom as well as a doublet signal that displays a smaller coupling constant to the phosphorus atom. The only obvious differences are the reversed relative chemical shifts of the protons *cis* and *trans* to the phosphorus atom (arrows) as well as reversed positions of the aforementioned doublet and the resonance from the glycine α protons (asterisks). Furthermore, the synthetic and natural products were found to have identical masses as determined by high-resolution Fourier-transform mass spectrometry, indicating identical atomic compositions, and both compounds showed fragmentation patterns consistent with a Gly-Leu dipeptide fragment. The structure of synthetic **2** was confirmed unambiguously by X-ray crystallography (see the Supporting Information), and therefore the previously reported structure of A53868 must necessarily be reassigned.

Given the identical masses of the synthetic and natural products, one possibility that was explored involved changing the order of the Gly and Leu residues. This peptide was prepared analogously to the route described for **2**, but NMR spectroscopy clearly showed it was not A53868 (not shown). Based on the previously reported NMR spectroscopy data,[11] two candidate isomeric structures **10** and **11** were envisioned.



Since crystallization attempts with the natural product to confirm its structure were unsuccessful, the producing organism was grown on minimal media with $(^{15}NH_4)_2SO_4$ as the sole nitrogen source to probe whether **10** or **11** could be the structure of A53868.

The compound produced was purified, and its ^{31}P NMR spectrum was recorded. A clear doublet was observed with a coupling constant of 8.6 Hz. However, no coupling was observed in the ^{1}H NMR spectrum between the ^{15}N nucleus and the doublet at $\delta = 3.3$ ppm corresponding to the putative methylene protons, thus ruling out structure **11**. Furthermore, the coupling constant of 8.6 Hz between the ^{31}P and ^{15}N nuclei appeared small for compound **10**. Since we could not find any literature values for the coupling expected for a structure like **10**, the producer organism was subsequently grown on minimal media with $(^{15}NH_4)_2SO_4$ as the only exogenous source of nitrogen and with [$^{13}C_6$]glucose as the carbon source to allow characterization by ^{13}C NMR spectroscopy. The NMR spectrum of the purified and uniformly ^{15}N,^{13}C-labeled A53868 displayed the expected splitting patterns for the vinyl carbons due to interaction with each other and couplings to the phosphorus and nitrogen atoms. Unexpectedly, however, the putative allylic methylene carbon atom was still present as a doublet, showing only the splitting from the phosphorus atom that is seen in unlabeled A53868, while no splitting from ^{13}C or ^{15}N nuclei was detected. These observations are incompatible with either structures **10** or **11** as candidates for A53868. Our data require that the putative methylene carbon atom cannot be directly attached to nitrogen, phosphorus, or carbon atoms.

The assignment of a methylene group to the signal at $\delta =$ 3.3 ppm for the proton and $\delta = 52$ ppm for the ^{13}C NMR spectra in the original work was based on the multiplicity of the signal in the ^{13}C NMR spectrum (reported as a triplet).[11] However, our ^{13}C NMR spectroscopy data indicate that this signal is in fact a methyl group.[14] On the basis of this assignment, data from ^{13}C,^{13}C COSY, HMBC, and HMQC NMR spectroscopy experiments, and the splitting patterns observed in the ^{13}C NMR spectrum of uniformly ^{15}N,^{13}C-

labeled A53868, we conclude that the structure of A53868 is compound **3**, in which a Gly-Leu dipeptide is attached to the monomethyl ester of a phosphonic acid analogue of dehydroalanine. This structure explains all spectroscopic observations including some diagnostic observations. First, in the ^{13}C NMR spectrum, the peak corresponding to the methyl ester carbon atom is split by phosphorus ($^2J(P,C) = 5.3$ Hz) in the unlabeled compound, and as expected on the basis of structure **3**, no additional couplings are observed in the ^{15}N- and ^{13}C-labeled analogue. Second, in the latter material, the quaternary vinyl carbon atom exhibits a doublet of doublet of doublets with splitting from the phosphorus atom ($^1J(P,C) = 190$ Hz), the methylene vinyl carbon atom ($^1J(C,C) = 73$ Hz), and the nitrogen atom ($^1J(N,C) = 11$ Hz; Figure 2). Third, the

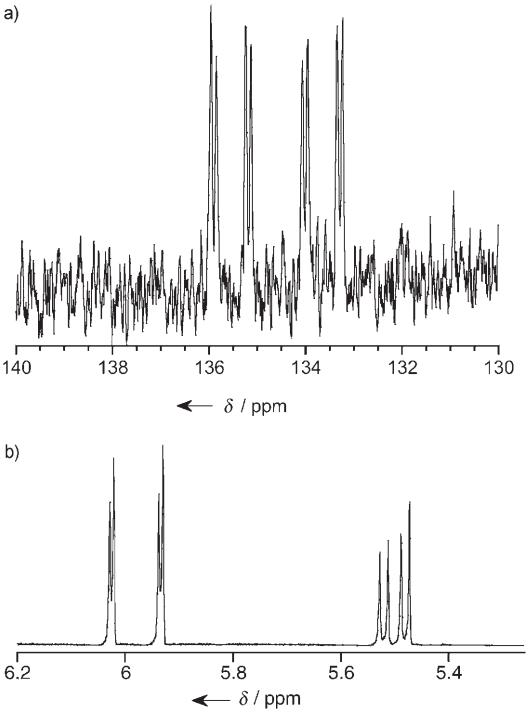
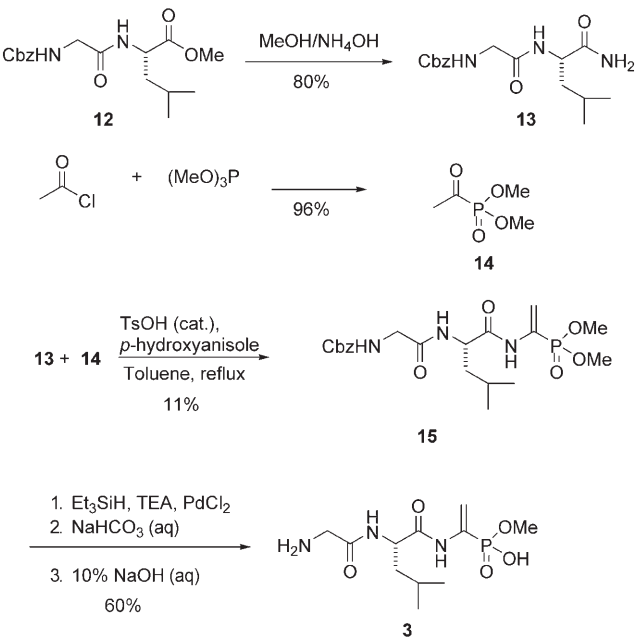


Figure 2. a) Vinyl carbon signal in the ^{13}C NMR spectrum of uniformly ^{15}N- and ^{13}C-labeled A53868. b) ^1H NMR spectrum showing the vinyl protons in the same compound.

signal from the carbonyl carbon atom of the Leu residue in A53868 exhibits a coupling constant of 14 Hz as a result of splitting by the ^{15}N nucleus in uniformly ^{15}N-labeled A53868, and finally in the ^1H NMR spectrum of this latter compound, the two vinyl protons display the expected couplings for protons *cis* and *trans* to a ^{15}N and ^{31}P nucleus (Figure 2; $^3J(P,H_{cis}) = 15.7$ Hz; $^3J(P,H_{trans}) = 36.3$ Hz; $^3J(N,H_{cis}) = 2.8$ Hz; $^3J(N,H_{trans}) = 6.1$ Hz).

To confirm our reassignment of structure **3** to A53868, the compound was prepared as shown in Scheme 3 by the acid-catalyzed condensation of dipeptide **13** with α-keto phosphonate **14** to form **15**.[15] This compound was highly reactive and was immediately carried through by transformation of the carboxybenzyl group to a carboxy(triethyl)silyl group.[16] Subsequent hydrolysis afforded the free amine without



Scheme 3. Synthesis of A53868 (**3**). Ts = 4-toluenesulfonyl, TEA = triethylamine.

affecting the vinylphosphonate. Basic hydrolysis of the phosphonate diester to the monoester[17] yielded the target compound, which was spectroscopically identical to that of the natural product (Supporting Information), and the compounds had identical fragmentation patterns in their mass spectra.

The reassignment of the structure of A53868 raises interesting questions with respect to its biosynthetic pathway as well as its mode of action. It is generally believed that the peptidic nature of other phosphonate antibiotics such as phosphinothricin tripeptide (PTT or bialaphos), alafosfalin (also called alaphosphin), and the plumbemycins promotes the uptake by the target organism(s) mediated by peptide permeases. Upon translocation, these peptides are hydrolyzed to release the active phosphonate-containing amino acids that target cellular enzymes such as glutamine and glutamate synthetase (phosphinothricin),[18,19] alanine racemase (alafosfalin),[20,21] or threonine synthase (plumbemycin).[22] Hydrolysis of the C-terminal peptide linkage of A53868 would result in an enamine that is expected to hydrolyze to methyl acetylphosphonate, which is a structural analogue of pyruvate and could inhibit pyruvate-utilizing enzymes.[23] On the other hand, the reactive vinyl phosphonate moiety in A53868 may inhibit a cellular process directly, analogous to the activity of the phosphonotripeptide K-26, which inhibits angiotensin-converting enzymes without the requirement of hydrolysis.[24] Interestingly, although the originally proposed structure **2** for A53868 is shown here to be incorrect, the synthetic compound **2** did have antimicrobial activity against *E. coli*.

In summary, the structure of A53868 was reassigned on the basis of spectroscopic analysis of isotopically labeled compound which was purified from the native producer as well as by chemical synthesis. The compound contains a phosphonate analogue of dehydroalanine, which is found in

many natural products such as the lantibiotics,[2] microcystins,[25] and thiostrepton.[26] On the basis of the revised structure **3**, we propose to name the compound dehydrophos. Its biosynthesis and mode of action are currently under investigation.

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