Penilumamide, a novel lumazine peptide isolated from the marine-derived fungus, *Penicillium* sp. CNL-338†

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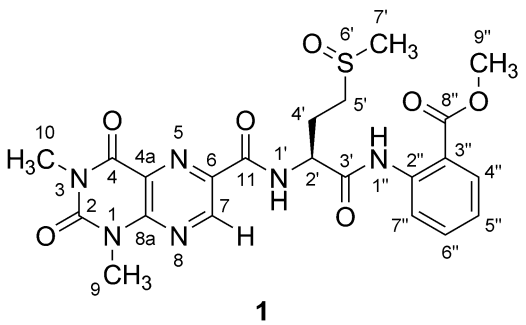
A novel lumazine peptide, penilumamide (**1**), was isolated from the fermentation broth of a marine-derived fungal strain, identified as *Penicillium* sp. (strain CNL-338) and the structure of the new metabolite was determined by analysis of ESI-TOF MS data combined with 1D and 2D NMR experiments.

1. Introduction

During the past decade, on average approximately 700 novel marine natural products have been published each year[1] of which 16–18% were of microbiological origin.[2] Therefore, marine microorganisms are an important resource for novel natural products.[3] Emerging as a significant component of the marine microbiota are marine-derived fungi, which, like their terrestrial counterparts, are a prolific source of unusual metabolites.[4] In a screening program, using the human colon tumor cell-line HCT-116, the extracts of more than 500 marine-derived fungal strains were examined. One strain, labelled CNL-338, identified as a *Penicillium* sp.,[5] attracted attention because the culture extract showed low micromolar cancer cell cytotoxicity. Bioassay-guided fractionation illustrated that the active compounds were the known cytotoxins aspochalasins D[6] and E.[7] However, during this fractionation a unique yellow metabolite was isolated that had striking structural features.

In this paper, we report the structure of penilumamide (**1**, Scheme 1), a peptide with an unusual starter unit, 1,3-dimethyllumazine-6-carboxylic acid, coupled to methionine sulfoxide and anthranilic acid methyl ester. A 1,3-dimethyllumazine-6-carboxamide moiety within a natural product was only published once[8] whereas the "monomers" 1,3-dimethyllumazine-6-carboxylic acid and 1,3-dimethyllumazine-6-carboxamide are unknown as natural products.



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Scheme 1 Chemical structure of penilumamide (**1**) isolated from *Penicillium* sp. CNL-338.

2. Results and discussion

Penilumamide (**1**) was obtained as a yellow powder that analyzed for the molecular formula $C_{22}H_{24}N_6O_7S$ ([M+Na]$^+$ m/z = 539.1272).[9] The presence of pseudomolecular adduct ions [M+Li]$^+$, [M+H]$^+$, [2M+H]$^+$, and the corresponding ions identified in negative ion mode, further verified the proposed molecular formula. Interpretation of NMR spectral data confirmed the presence of 22 carbon atoms, 24 protons and 6 nitrogen atoms (see Table 1). Four methyl groups were observed, which by their chemical shifts were all bound to heteroatoms (δ_H 3.68, 3.60, 3.37 and 2.57 ppm). Furthermore, two methylene groups, five aromatic/olefinic methine protons and five singlet resonances, ascribable to either carbonyl groups or other related carbons, were observed (δ_C 169.4, 167.3, 163.0, 159.2 and 150.4 ppm). By interpretation of ^{1}H-NMR coupling patterns and the analysis of 2D NMR experiments, a methyl ester of anthranilic acid and a methionine sulfoxide moiety could be unambiguously assigned. The latter substructure showed the same NMR chemical shifts as commercially available methionine sulfoxide (Sigma, Fluka). For the undefined part of the molecule, a *N*-rich and highly unsaturated moiety remained ($C_9H_7N_4O_3$, 8 degrees of unsaturation). A part of this structural element (C8a-N1-C2-N3-C4), including two *N*-bound methyl groups (δ_H 3.60 and 3.37 ppm), was assigned *via* interpretation of standard 2D NMR experiments. The structure of penilumamide (**1**) was further predicted using the NMR-based structure generator Cocon (Table 2).[10] For this purpose, different NMR correlation data sets were investigated with respect to the number of possible constitutional proposals.

While a Cocon input file including all available NMR correlation information generated 212 possible structures, the number increased to 404 if the 1,1-ADEQUATE correlation data were omitted. The incorporation of ^{1}H,^{15}N-HMBC data proved to be even more important: a data set without these correlations resulted in 1869 structures and a set using solely ^{1}H,^{1}H-COSY and

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Table 1 NMR data of penilumamide (**1**, 400 MHz, DMSO-d_6 in ppm)[a]

Pos.	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^{15}N)$
1	—	—	134
2	—	150.4	—
3	—	—	163
4	—	159.2	—
4a	—	126.1	—
5	—	—	333
6	—	139.3	—
7	9.32 (d, $J = 9.2$ Hz)[b]	146.8	—
8	—	—	304
8a	—	149.5	—
9	3.60 (s)	29.4	—
10	3.37 (s)	28.6	—
11	—	163.0	—
1′	9.31 (s)	—	121
2′	4.85 (ddd, $J = 13.2$, 9.2, 4.8 Hz)	53.3/53.6[c]	—
3′	—	169.4	—
4′	2.30–2.39 (m)	23.8/24.2[c]	—
	2.42–2.49 (m)		
5′	2.73–2.81 (m)	49.7/49.9[c]	—
	2.88–2.97 (m)		
7′	2.57 (s)	37.9/38.1[c]	—
1″	11.06 (s)	—	133
2″	—	139.0	—
3″	8.33 (d, $J = 7.9$ Hz)	121.1	—
4″	7.63 (ddd, $J = 7.9$, 7.5, 1.5 Hz)	134.0	—
5″	7.21 (dd, $J = 7.5$, 7.0 Hz)	123.6	—
6″	7.90 (dd, $J = 7.0$, 1.5 Hz)	130.6	—
7″	—	117.8	—
8″	—	167.3	—
9″	3.68 (s)	52.3	—

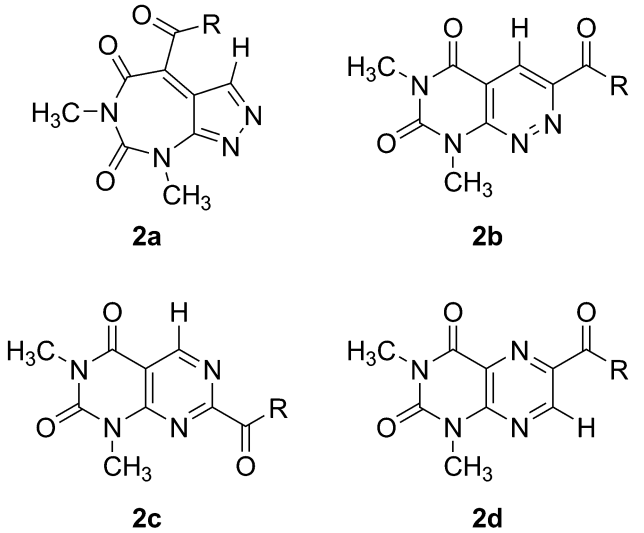
[a] ^1H and ^{13}C chemical shifts are referenced to the DMSO-d_6 signal (2.50 ppm and 39.5 ppm respectively). ^{15}N chemical shifts were not calibrated with an external standard. Therefore, the δ value has an accuracy of about 1 ppm in reference to NH_3 (0 ppm) and the ^{15}N chemical shifts are given without decimals. [b] The coupling constant was extracted from 1D ^1H-NMR spectrum in $CDCl_3$ in which H-7 and H-1′ are not overlapped, $\delta(^1H)$ 9.45 ppm (H-7), 9.18 ppm (H-1′). [c] Indicates diastereomers at chiral S^*.

Table 2 Results of the COCON calculations for penilumamide (**1**)

Data Set	COSY	$^1H,^{13}C$-HMBC	1,1-ADEQ	$^1H,^{15}N$-HMBC	Number of possible structures
A	X	X			3382
B	X	X	X		1869
C	X	X		X	404
D	X	X	X	X	212

$^1H,^{13}C$-HMBC correlations yielded 3382 possible constitutions (see Table 2).

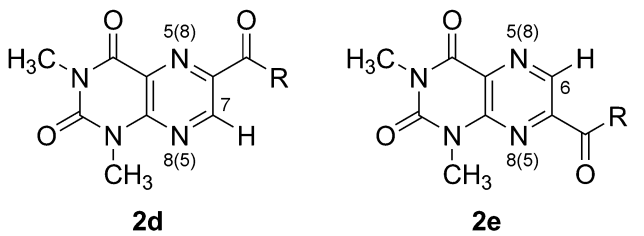
For the final structural assignment of the remaining part of this heterocycle, the application of 1,1-ADEQUATE and $^1H,^{15}N$-HMBC experiments were essential. Starting from the COCON results, two structural alternatives were conceivable: a 5/7-bicyclic ring system (**2a**) and two condensed six-membered rings (**2b–d**) (all Scheme 2), both structural alternatives are possible in alternative constitutions. The ^{13}C- and ^{15}N-NMR chemical shifts, as well as considerations reflecting the likely biosynthesis, argued against the 5/7-bicyclic ring system (**2a**). From the three isomers 1,2-aza (**2b**), 1,3-aza (**2c**) and 1,4-aza (**2d**) the lumazine-like 1,4-aza constitution was unambiguously confirmed by the combination of exactly one 1,1-ADEQUATE correlation and two $^1H,^{15}N$-HMBC correlations



Scheme 2 Structure elucidation of the heterocyclic remainder ($C_9H_7N_4O_3$) of penilumamide (**1**). The 1,4-aza variant (**2d**) is the constitution suggested for **1**.

from the single hydrogen of the 1,3-dimethyllumazine moiety (H-7 in **2d**).

With this assumption, two aspects of the structure had to be considered: a) the orientation of C-6 and C-7 (different isomers, Scheme 3) and b) the orientation of N-5 and N-8 (which would not change the constitutional formula, as seen in Scheme 3). UV and NMR data could not differentiate between these two.



Scheme 3 Isomeric structures **2d** and **2e**.

The HMBC correlation H-7/C-8a was more intense than the HMBC correlation H-7/C-4a. Thus arguing for the latter being a $^4J_{CH}$ crosspeak. A $^{2/3}J_{CH}$ correlation to C-4a was not observed, as would be required in **2e**. UV spectral data further assisted in assigning the proposed chemical composition.[11] Primarily, the values for the absorption maxima (1,3-dimethyllumazine-6-carboxylic acid, λ_{max} = 248 and 334 nm compared to 1,3-dimethyllumazine-7-carboxylic acid, λ_{max} = 243 and 349 nm)[11a] correlated well with the absorption maxima of penilumamide (**1**, λ_{max} = 223, 250, 319 and 334 nm). This is consistent with the carbonyl substitution at the 6-position.[9] Furthermore, the UV absorption spectra of **1** (Fig. 1) in neutral, basic and acidic methanol are almost identical. A comparison with the shifts of the bands within the spectra of synthetic leucettidine at pH 6 and 11 support position 6 as the site of carbonyl substitution.[12] On the basis of UV and NMR data, a C-6/C-7 orientation as shown in **2d** was assigned.

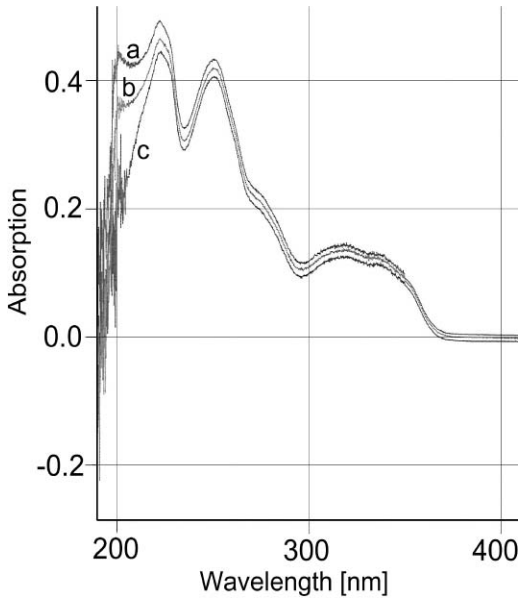


Fig. 1 UV spectra of penilumamide (**1**) in a) acidified MeOH, b) pH-neutral MeOH and c) basified MeOH.

The orientation of N-5 and N-8 can also be defined by $^{1}H,^{15}N$-HMBC correlation data, once the position of the H-6/H-7 had been defined. The $^{2}J_{NH}$ coupling constants in the lumazine system showed a similar *S*/*N* ratio as the crosspeak H-7/N-8, while the peak H-7/N-5 is substantially less intense.[9] Furthermore, a $^{4}J_{NH}$ correlation from H-1′ to N-5 (*c in Fig. 2) and a $^{4}J_{NH}$ correlation from H-9 to N-8 (*b in Fig. 2) were observed, favouring the isomer with N-8 being bound to C-7 (**2d**). The observed ^{13}C-NMR shifts were mostly in good agreement with those of commercially available lumazine (Aldrich)[9] or with other synthesized or isolated lumazine derivatives.[8,13]

To confirm the amino acid configurations of **1**, the compound was hydrolysed and the amino acids obtained were converted to their 1-fluoro-2,4-dinitrophenyl-5-L-alanine derivatives following Marfey's method.[14] The resulting methionine sulfoxide amino acid derivative was compared by HPLC with the two diastereomeric products prepared from D- and L-methionine sulfoxide.[15] Using this method, the methionine sulfoxide derivative incorporated in penilumamide (**1**) was established as L. The retention time for the FDAA-L-methionine sulfoxide product is 13.75 min and for the FDAA-D-methionine sulfoxide product it is 13.40 min.

In an attempt to determine the configuration of the sulfur atom we performed several NMR experiments with (*S*)- and (*R*)-α-methoxyphenylacetic acid (MPAA)[16] in order to see defined shift differences against the configuration of the sulfur atom due to complexation effects. The results of these experiments were ambiguous because the effects of MPAA on the ^{1}H chemical shifts in the spectra were too small and showed no pattern driven by the chirality of MPAA.[9] Attempts to determine the configuration of the sulfur atom *via* the crystal structure of tris(indenyl)lanthanium(III)–tetrahydrofuran adducts also failed.[17] Regarding the ^{13}C NMR data, the occurrence of a doubling of signals of the methionine sulfoxide moiety indicated the presence of a mixture of the *R* and *S* sulfoxides.[18] Comparing the ^{13}C NMR shifts of *R* and *S* sulfoxide yielded a ratio of 5 : 1, thus the sulfoxide to be 83% *S*

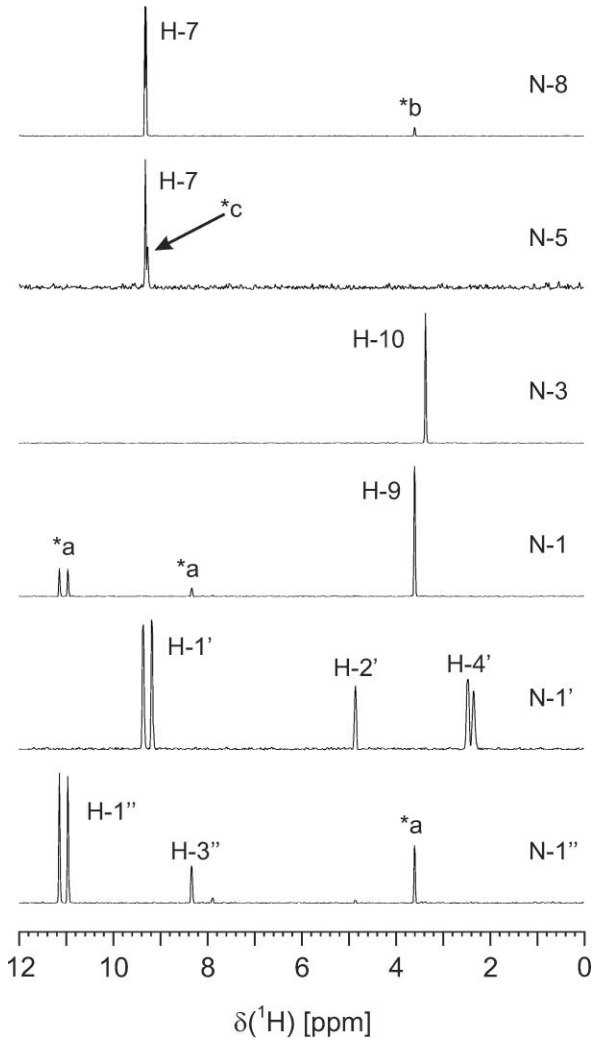
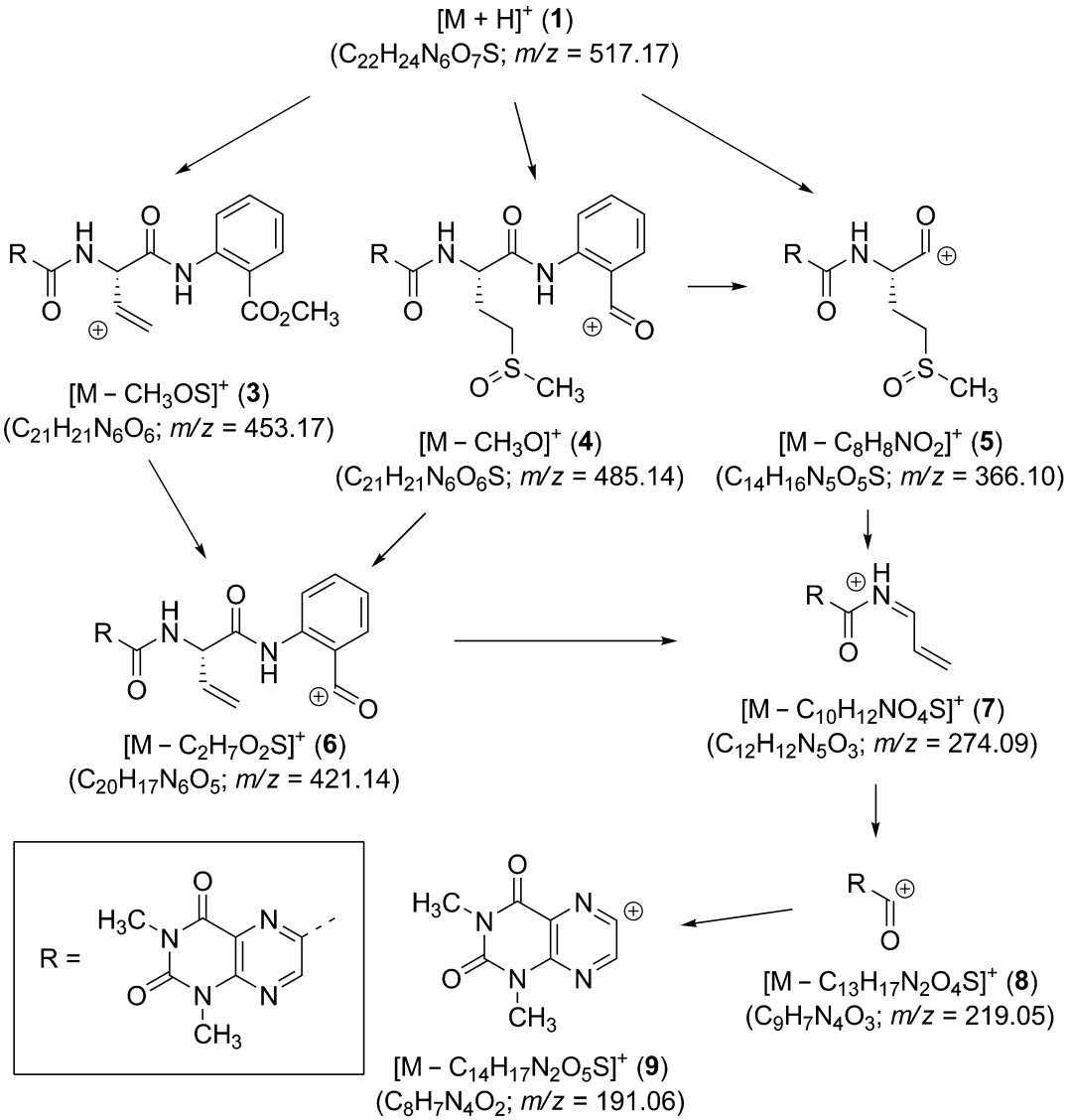


Fig. 2 Rows of all six nitrogens (N-1, N-3, N-5, N-8, N-1′ and N-1″) from the $^{1}H,^{15}N$-HMBC spectrum of **1**. *a indicates peaks which are not real peaks. In this case they appear because the chemical shifts of N-1 and N-1″ differ only by 1 ppm (which is just one row in the spectrum). *b is the $^{4}J_{NH}$ correlation from H-9 to N-8 and *c (less intense peak next to H-7) is the $^{4}J_{NH}$ correlation from H-1′ to N-5.

configurated.[19] When mentioned in the literature the *R*/*S* ratio of a methionine sulfoxide moiety within a cyclic peptide is 1 : 1.[18a,b,20]

Using HPLC-ESI-TOF MS under API-CID conditions and an ESI ion trap, MS/MS and MS^{n} experiments were carried out to verify the structural elements through interpretation of fragmentation patterns. Scheme 4 presents the observed ion peaks and a proposed interpretation. Starting with protonated penilumamide (**1**), elimination of a methyl sulfoxide group (**3**), demethoxylation of the ester group (**4**) or cleavage of the peptide bond bound to the benzene ring system (**5**) seems to occur. Within the next fragmentation steps the remaining moiety decomposes further to the final 1,3-dimethyllumazine heterocyclic system (**9**).

While the previously mentioned aspochalasins from CNL-338 were cytotoxic, penilumamide (**1**) showed no activity in a cytotoxicity assay. The structural similarity of the lumazine part of **1** to riboflavin led to the assumption that it could exhibit an antimicrobial activity. But no effect on the growth of *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* or



Scheme 4 Proposed MS/MS and MS^n fragmentations of penilumamide (**1**).

Candida albicans was observed. Penilumamide (**1**) showed no activity against three further Gram negative and three Gram positive bacteria as well as three yeasts and three fungi strains. Furthermore, no influence on the Ca^{2+} level was detectable in a test for the cellular Ca^{2+} signaling in neuroendocrine cells (PC12).[21]

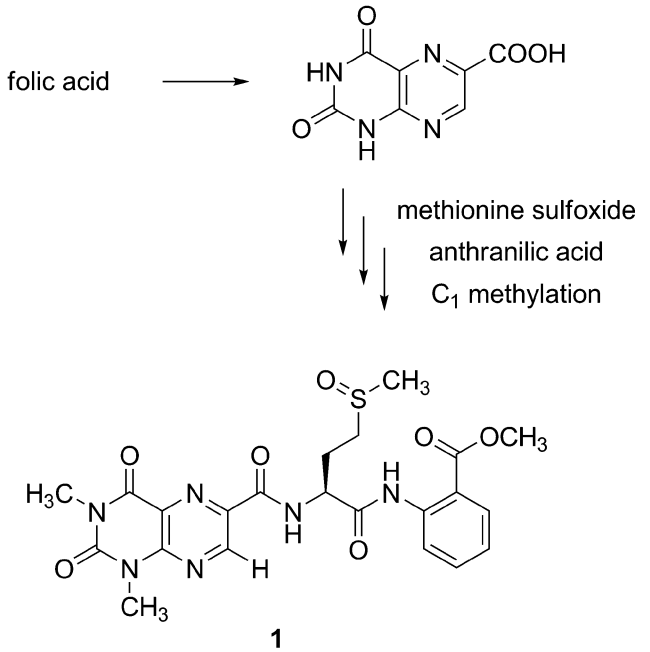
3. Conclusion

In summary, we have isolated and established the structure of a novel lumazine peptide through the application of a diversity of NMR and MS experiments. Our work results in the assignment of a structure to penilumamide (**1**) that combines a lumazine system with L-methionine sulfoxide and anthranilic acid ester. Although lumazines are well-known metabolites, this is the first example of the unique combination of a 1,3-dimethyllumazine-6-carboxylic acid, a methionine sulfoxide group and an anthranilic acid methyl ester moiety within a natural product. A discussion of the proposed biosynthetic pathway that is shown in Scheme 5 starts with folic acid that is well-known to undergo microbial degradation to lumazine-6-carboxylic acid.[22] A viable pathway then could include amino acid synthesis with the stepwise addition of methionine sulfoxide and lastly anthranilic acid. Penilumamide (**1**) could then be produced by multiple C1 methylations at the N-1, N-3, and C-8′ acid positions. Of course, many other pathways may be in place, but this proposal seems the most direct.

4. Experimental details

4.1 General experimental procedures

All NMR spectra were recorded using *Bruker* Avance 400 and 500 MHz spectrometers. $^1H,^1H$-COSY, $^1H,^{13}C$-HSQC, $^1H,^{13}C$-HMBC, $^1H,^{15}N$-HSQC, $^1H,^{15}N$-HMBC and 1,1-ADEQUATE experiments were carried out at 300 K using standard parameters. The delay set for HMBC spectra acquisition was set to 80 ms (^{13}C)



Scheme 5 Proposed biosynthesis of penilumamide (**1**).

and 120 ms (^{15}N), respectively. The coupling constants chosen for the 1,1-ADEQUATE-spectrum were 180 Hz (J_{CH}) and 55 Hz (J_{CC}), and the multiplicity selection was set to CH.

High resolution mass spectra were recorded with an ESI-TOF mass spectrometer (*Bruker* micrOTOF$_{LC}$). MS/MS and MS^n experiments were performed using an ESI ion trap (*Bruker* Esquire 3000plus) and an HPLC-ESI-TOF MS (*Agilent* 1100 HPLC system and *Bruker* micrOTOF$_{LC}$) under API-CID conditions. For mass calibration a sodium formate cluster was used.

FT-IR spectra were recorded as KBr pellets at room temperature using a *Bruker* Equinox55 spectrometer with DTGS detector. Fifty scans were acquired for each spectrum. UV spectra were recorded with a *Varian* spectrometer type Cary 3E and with a diode array detector during HPLC analysis. The optical rotation value was determined with a *Perkin-Elmer* polarimeter.

4.2 Fermentation and isolation

The fungal strain CNL-338 was isolated from the red alga *Laurencia* sp. collected in the Bahamas Islands. The strain was cultured in nine 2.8 L Fernbach flasks each containing 1 L of the marine-based fermentation medium YPG+C (0.5% yeast extract, 0.5% peptone, 1% glucose, 0.2% crab meal, 100% seawater). The flasks were shaken at 230 rpm for 23 days and the whole culture broth was extracted with 9 L of ethyl acetate. The dried EtOAc extract was then subjected to Waters Sep-Pak (20 mL, 5 g C18 cartridge) fractionation eluting stepwise with MeOH–H_2O mixtures. The fractions containing penilumamide (**1**) were further purified *via* isocratic HPLC (semi-preparative, Phenomenex Luna 5 μm RP-C18, 10 × 250 mm, 4.5 mL/min) using MeOH–H_2O (8 : 2) yielding the pure compound **1** (15 mg).

Penilumamide (**1**) was obtained as a yellowish powder. UV (MeOH): λ_{max} = 223, 250, 319, 334 nm, see Fig. 1; IR (KBr): ν = 3517, 2957, 1724, 1679, 1605, 1586, 1549, 1501, 1450, 1380, 1290, 1266, 1188, 1142, 1089, 1032, 1013, 962, 814, 749, 702, 490, 451, 415 cm^{-1}; $[\alpha]_D^{20}$ = +49.0 (*c* 0.29, MeOH); HR(+)ESI-MS: m/z = 1055.2754 $[2M+Na]^+$ (calcd. for $C_{44}H_{48}N_{12}O_{14}S_2Na$ 1055.2747), $\Delta m/z$ = 0.7 ppm; m/z = 539.1333 $[M+Na]^+$ (calcd. for $C_{22}H_{24}N_6O_7SNa$ 539.1319), $\Delta m/z$ = 2.6 ppm; m/z = 523.1583 $[M+Li]^+$ (calcd. for $C_{22}H_{24}N_6O_7SLi$ 523.1582), $\Delta m/z$ = 0.3 ppm; HR(-)ESI-MS: m/z = 1031.2773 $[2M-H]^-$ (calcd. for $C_{44}H_{47}N_{12}O_{14}S_2$ 1031.2771), $\Delta m/z$ = 0.2 ppm; m/z = 515.1338 $[M-H]^-$ (calcd. for $C_{22}H_{23}N_6O_7S$ 515.1343), $\Delta m/z$ = 1.0 ppm; ^1H- and ^{13}C-NMR data, see Table 1.

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