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Synthesis and bioactivity of nobilamide B

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Abstract

An alternative and facile solution/solid-phase approach is reported for the total synthesis of neuroactive peptide, nobilamide B. Z-Dhb was formed in solution via EDC/CuCl induced elimination. The solid-phase synthesis employed HBTU/Oxyma PureTM coupling using Barlos resin. Synthetic nobilamide B was also found to be neuroactive in primary cultures of dorsal root ganglion (DRG) neurons.

Lin et al. reported a family of peptides, coined the nobilamides, which were isolated from *Streptomyces* sp. cultured from tissues of mollusks, *Chicoreus nobilis*.¹ Nobilamide B (**1**) exhibited neuroactivity based on its inhibition of the action of capsaicin on TRPV1, implicated in inflammation and sensation of pain.

Nobilamide B (**1**)

The synthesis of **1** was first described by Yamashita and co-workers² using Staudinger ligation to generate the didehydrobutyryne residue in tandem with solid-phase peptide synthesis. In this study, we report an alternative, more flexible and facile synthesis of **1** which can be used for the synthesis of structural analogues.

[†]Electronic Supplementary Information (ESI) available: Analytical HPLC profile, NMR and ESI-Mass Spectra are included in the supplementary information. See DOI: 10.1039/c000000x/

In designing the synthetic strategy to **1**, synthesis of the non-proteinogenic amino acid Z-Dhb was first accomplished prior to conventional solid phase synthesis of the complete peptide. Many different approaches to synthesize dehydroamino acids have been reported but elimination is the most described.³ Dehydration of the unprotected activated -OH group is the direct method for the synthesis of dehydroamino acids especially if the β -hydroxy amino acid precursors, such as serine and threonine, are available.⁴⁻⁶ Reports of various reagents used for the activation of the hydroxyl group and elimination with an appropriate base have grown extensively. Jimenez, et.al reported a carbodiimide activation of the hydroxyl group using EDC in the presence of CuCl in both solution and solid-phase methods, where only the Z isomer (the most thermodynamically stable) is formed.⁵ Almost quantitative conversion is observed using the EDC/CuCl system in the preparation of peptides involving L-Ser, L-Thr (including D-allo-Thr), and L/D-(β -OH)Phe. Didehydroamino acids should not be generated prior to coupling with other amino acids because the carboxylic acid of a didehydroamino acid couples less efficiently than a regular amino acid. Similarly, the presence of the double bond decreases the nucleophilicity of the amine group.^{5,7}

Synthesis of Z-Dhb (Scheme 1) was achieved by the conversion of *tert*-butyl C-terminal protected threonine, H-Thr-O^tBu·HCl (**2**) to H-Thr-O^tBu (**3**) and coupling with Fmoc-Ala-OH (**4**) in a carbodiimide based reaction in dichloromethane under basic conditions to produce Fmoc-Ala-Thr-O^tBu (**5**) with 89% yield. Elimination of the -OH of **5** involved a carbodiimide mediated dehydration using EDC in the presence of CuCl via E₁ or E_{1cb} mechanism⁵ similar to the method of preparation of didehydrobutyrine reported for the synthesis of Kahalalide F⁸ and cyclic liponadepsipeptide [*N*-Mst(L-Ser1), D-Ser4, L-Thr6, L-Asp8, L-Thr9]syringotoxin.⁷

Conversion of **5** to **6** selectively produced the Z-isomer of the alkene with 72 % yield. Dehydration reaction can produce either a Z or E isomer or a mixture of both depending on the conditions and reagents. Even though literature sources⁵ confirm that the reagents used in the dehydration reaction selectively produces the Z-isomer, a 1D NOE experiment (Figure 1) was performed in order to determine the stereochemical configuration of the double bond. By irradiating the quartet proton signal at 6.73 ppm, which corresponds to Dhb methine proton, the stereochemistry can be confirmed by identifying which of the signals has an increase in intensity.

Selective excitation of the proton at 6.73 ppm induces an increase in signal for the proton resonances at 1.73 and 1.48 ppm (Figure 1b). The increase of the signal in 1.73 ppm corresponds to the interaction of the methine proton with the methyl protons of the Dhb due to their proximity. The increase of the signal at 1.73 ppm is more evident for the methyl protons (ten-fold greater) than the increase in the signal of methyl protons of *tert*-butyl group at 1.48 ppm because the methyl protons are closer to the methine proton of Dhb relative to the *tert*-butyl protons. This clearly shows that the methine proton is close to the *tert*-butyl protons of the C-terminal protecting group due to the increase of signal in 1.48 ppm and verifies that the isomer formed is a Z-isomer.

To further support the claim that the Z-isomer was formed, a 1D NOE spectrum was obtained wherein the selective excitation band chosen was at 1.73 ppm for the *tert*-butyl protons (Figure 1c). If the alkene was an *E*-isomer, irradiating the signal at 1.73 ppm should increase the signal at 1.48 ppm. Clearly, there is no increase in signal observed in the spectrum. Therefore, the stereochemical configuration of the alkene generated is exclusively the Z-isomer.

The formation of Fmoc-Ala-Z-Dhb-OH (**7**) from **6** involved a simple deprotection of the *tert*-butyl protecting group of the C-terminal of didehydrobutyrine via A_{AL}1 elimination reaction⁹ using high concentrations of TFA with 90% yield. The dipeptide **7** successfully conjugated to the Barlos resin using a slight excess of **7** (1.1 equiv) in the span of 1.5 h. The attachment of the dipeptide was confirmed by the Kaiser test, quantified using Fmoc assay^{10,11} and calculated to have 1.67 mmol of **7** per g of resin (98% loading). After the addition of the dipeptide **7** to the Barlos resin, conventional solid phase peptide synthesis was employed to yield **1** with 92% yield, >40 % purity. The peptide **1** was then purified using semi-preparative HPLC before characterization to obtain an overall yield of 25% with >98% purity. The peptide was characterized by HRMS with a peak at *m/z* 858.5299 calculated for the [M+Na]⁺.

The isolated and synthetic **1** were co-eluted and the resulting chromatogram showed only a single peak (E.S.I). Hence, the solution behaviour of the synthetic peptide is identical with that of the isolated.

The bioactivity of the synthetic compound was evaluated in the DRG assay. The primary cultures of DRG contain at least 12 definable cell types that express distinct receptor and channel populations, including a substantial fraction of nociceptors (~30-50%).¹²⁻¹⁵ Using calcium imaging, we routinely utilize this broad scope assay to screen for neuroactive compounds.

The assay result showed that synthetic nobilamide B inhibited the response of DRG neurons to capsaicin (Figure 2). This is similar to the result obtained with the isolated natural product, which induced an inhibitory effect to TRPV1.

Conclusions

Nobilamide B (**1**) was successfully synthesized and found to be structurally identical to the isolated natural product. ¹H- and ¹³C-NMR experiments validate the stereochemical assignment (absolute configuration) of the natural product. HPLC coelution demonstrates that the synthetic and isolated peptides have the same solution behaviour. Furthermore, the DRG assay demonstrates that the synthetic compound is neuroactive with inhibitory effect on capsaicin. At present, we are employing the synthetic route described above for the production of analogues in order to conduct a comprehensive structure-activity relationship study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. 1D NOE Difference spectrum of (6) a) ^1H NMR spectrum, b) 1D NOE with selective excitation at 6.73 ppm; c) 1D NOE with selective excitation at 1.73 ppm.

Figure 2.

Activity of synthetic nobilamide B observed by calcium imaging of dissociated DRG neurons in culture. Each trace is a response of a single neuron. Responses from 100–150 neurons were monitored individually and simultaneously in a given experimental trial. The x axis is time in minutes and is the same for all traces in the given panel. The y axis is a measure of relative $[Ca^{2+}]$ determined by 488 nm excitation and fluorescent emission monitored at 516 nm. Response to capsaicin (observed as a peak in each trace) indicates the presence of TRPV1 receptors on neurons. Neurons were stimulated with 100 nM capsaicin for ~15 s (indicated by the arrow) before and after the test compounds were applied. Horizontal bars indicate the incubation (5 min). DMSO was used as blank test.

Scheme 1.
Synthesis of nobilamide B (**1**).

Table 1¹H chemical shifts of the isolated¹ and synthetic nobilamide B.

Residue		Chemical Shift, δ ppm (<i>J</i> value, Hz)	
		Isolated	Synthetic
Z-Dhb	3	6.58 q (7.0)	6.54 q (7.1)
	4	1.64 d (7.0)	1.60 d (7.1)
	NH	9.04 s	9.00 s
L-Ala	2	4.43 m	4.38 m
	3	1.31 d (7.0)	1.27 d (7.1)
	NH	8.19 d (7.7)	8.15 d (7.1)
L-Val	2	4.30 dd (6.5, 8.5)	4.25 m
	3	2.03 m	1.99 m
	4/5	0.86 d (6.0)/ 0.88 d (6.0)	0.82 d (6.0)/ 0.84 d (6.0)
	NH	7.75 d	7.70 d
D- <i>a</i> -Thr	2	4.36 dd (7.0, 7.2)	4.30 m
	3	3.83 m	3.79 m
	4	1.07 d (5.8)	1.02 d (6.3)
	NH	8.14 d (8.6)	8.08 d (8.5)
L-Phe	2	4.59 m	4.58 m
	3	3.06 dd/ 2.71 m	3.08 dd/ 2.70 m
	Ph	7.02-7.30 m	7.14-7.27 m
	NH	8.28 d (8.4)	8.29 d (8.5)
D-Leu	2	4.20 m	4.19 m
	3	1.15 m	1.15 m
	4	1.16 m	1.16 m
	5/6	0.73 d (5.6)/ 0.70 d (5.6)	0.73 d (5.9)/ 0.70 d (6.0)
	NH	7.92 d (8.0)	7.94 d (8.0)
D-Phe	2	4.48 m	4.48 m
	3	2.93 dd/2.69 m	2.94 dd/2.70 m
	Ph	7.0-7.3 m	7.14-7.27 m
	NH	7.91 d (8.0)	7.93 d (8.3)
Prop	2	1.98 m	1.99 m
	3	0.82 m	0.82 m