



Total synthesis of μ -conotoxin lt5d†

Cite this: *RSC Adv.*, 2018, 8, 36579

A. M. B. Naraga,^{id} O. J. V. Belleza^{id} and A. J. L. Villaraza^{*}

Received 30th April 2018
Accepted 23rd October 2018

DOI: 10.1039/c8ra03706j

rsc.li/rsc-advances

Liu *et al.* previously described the isolation and characterization of a neuroactive μ -conotoxin lt5d from *Conus litteratus*.¹ Such peptides are of interest since conopeptides isolated from *Conus* snail venoms have high specificity and activity towards neuronal ligand- and voltage-gated channels, receptors and transporters, making them potential peptidic drug candidates for neurodegenerative and chronic pain conditions.² For instance, one of which is the FDA approved ω -conotoxin MVIIA, commercially available as Prialt®, used for the management of severe chronic pain experienced by patients suffering from cancer, diabetes, multiple sclerosis, and HIV/AIDS.^{3–6} However, there are over 800 marine snails of genus *Conus* distributed throughout tropical and subtropical waters, each having approximately 50 to 200 conopeptides with only a few overlaps among species. Such biodiversity presents a rich source of novel and bioactive compounds.^{2,7}

Lt5d, a 12-residue conotoxin belonging to the T-superfamily with cysteine framework V, was previously reported to have dose-dependent inhibitory activity against tetrodotoxin (TTX)-sensitive Na channel receptors in adult dorsal root ganglion (DRG) neurons.^{1,8,9} However, the total chemical synthesis of lt5d has yet to be described. In this study, we report the total chemical synthesis of lt5d using two different peptide folding strategies.

Scheme 1 summarizes the first method employed (*i.e.*, Method 1). To achieve synthetic lt5d, the linear peptide was first synthesized on solid phase using *N*-Fmoc protected amino acids, in which the Cys residues were protected with the trityl (Trt) group. Afterwards, 1 mM oxidized glutathione (GSSG), together with equimolar amounts of reduced glutathione (GSH) and EDTA, were combined with a 20 μ M solution of linear peptide to yield the folded product.¹⁰ GSSG acts as the oxidizing agent while GSH acts as the reducing agent, since GSH is known for correcting misbridged cysteine residues of proteins in the

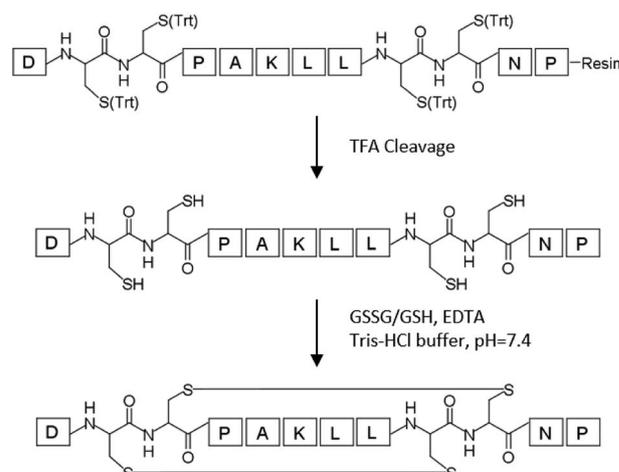
The total synthesis of μ -conotoxin lt5d is presented for the first time employing two different strategies. One involves glutathione-assisted oxidation where all disulphide linkages are formed simultaneously. Another involves orthogonal protection of cysteine residues, allowing the controlled formation of disulphide linkages sequentially. Both methods achieve the same peptide.

endoplasmic reticulum where proteins are folded *in vivo*.^{11,12} These conditions are widely adopted to mimic physiological conditions favouring the formation of thermodynamically stable folded proteins and cysteine-rich peptides *in vitro*.^{13–15}

Fig. 1 presents the HPLC chromatograms (C_{18}) of pure linear and folded lt5d. A shift in retention time from $t_R = 24.3$ min to 23.1 min is indicative of folding as the shape and the number of exposed residues change. This leads to less interaction with the hydrophobic C_{18} stationary phase and hence earlier elution of the folded peptide.

The identities of the products were confirmed by high resolution electron spray ionization mass spectrometry in positive mode (HR-ESI(+)-MS). Fig. 2 shows the $[M + H]^+$ molecular ion peaks at $m/z = 1279.6920$ and 1275.6353 which correspond to the calculated molecular weights of linear (1278.52 Da) and folded peptide (1274.49 Da), respectively. The 4 Da mass difference is due to the loss of four hydrogen atoms upon the formation of the two disulphide bridges.

However, Method 1 assumes that disulphide bonds Cys2–Cys9 and Cys3–Cys10 were formed in solution by glutathione



Scheme 1 Lt5d synthesis using glutathione-assisted oxidative folding (Method 1).

Institute of Chemistry, College of Science, National Science Complex, University of the Philippines, Diliman, Quezon City, 1100, Philippines. E-mail: avillaraza@up.edu.ph
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c8ra03706j



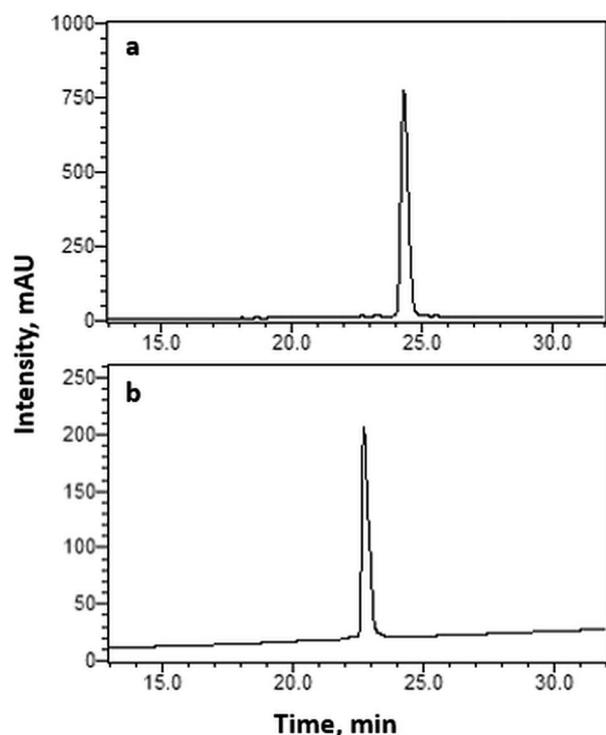


Fig. 1 RP-HPLC chromatograms of pure (a) linear and (b) folded lt5d, $t_R = 24.3$ min and $t_R = 23.1$ min, respectively. Analytical C_{18} column was used to profile the sample with a linear gradient from 15% to 45% solution B (90% MeCN in H_2O with 0.1% TFA) for 35 min at 1 mL min^{-1} flow rate. Absorbance was monitored at 220 nm.

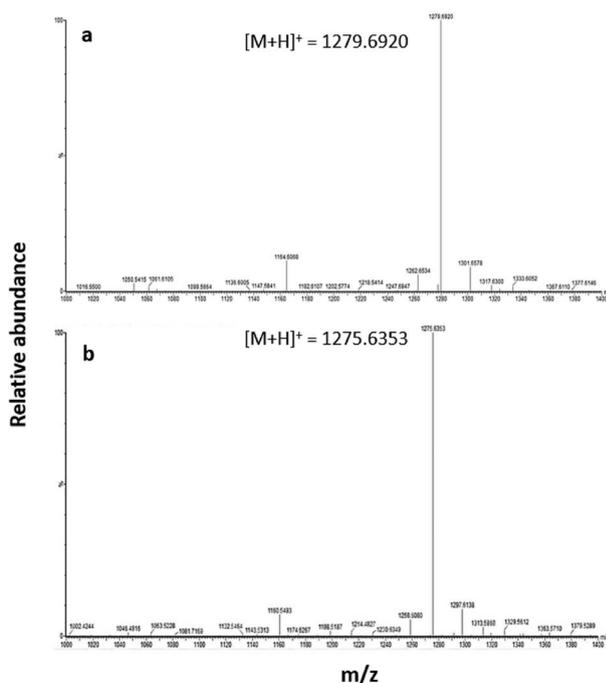
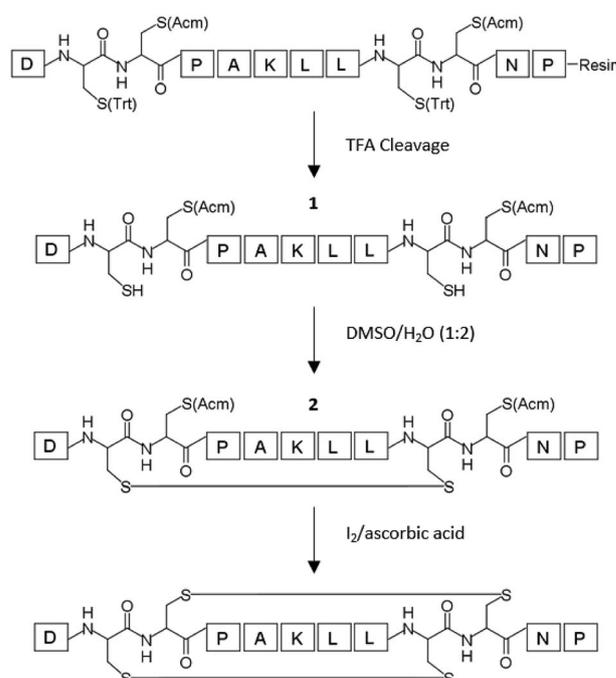


Fig. 2 HR-ESI(+) mass spectra of pure (a) linear lt5d with the molecular ion peak at 1279.6920, and (b) folded lt5d at 1275.6353. Calculated monoisotopic mass of target linear and folded peptide is 1278.5229 and 1274.4916 Da, respectively.

oxidation. In order to verify this, an alternative method in which the disulphide bonds are sequentially formed in a controlled manner by orthogonal cysteine protection was devised (*i.e.*, Method 2), as described in Scheme 2.^{16,17} In brief, two different protecting groups, trityl (Trt) and acetoxymethyl (Acm), were used in each pair of Cys residues. Upon acidolytic cleavage of the peptide from the resin, the Trt protecting groups were removed from Cys2 and Cys9. As the Acm protecting groups are resistant to TFA cleavage, Cys3 and Cys10 remained protected under these conditions. Since the HPLC chromatogram (C_{18}) of “crude” linear peptide **1** (Fig. 3a) showed a single peak at 21.39 min, the peptide was folded without further purification: linear peptide **1** was dissolved in 1 : 2 DMSO/water and the oxidative folding reaction stirred for 24 h. The reaction was quenched by addition of an equal volume of acetic acid to the reaction mixture, forming partially folded peptide **2**. The HPLC chromatogram (C_{18}) of **2** (Fig. 3b) showed a slight decrease in the retention time of the major product (21.14 min), indicating the formation of the first disulphide bond, Cys2–Cys9. In order to form the second disulphide bond Cys3–Cys10, the Acm protecting groups were cleaved using iodine. This reaction was carried out by adding a 5-fold excess of iodine to the solution, and quenched by the addition of ascorbic acid, yielding fully folded lt5d peptide, as evidenced by mass spectrometry and HPLC (Fig. 3c). In contrast with the previous observation that a shorter retention time is observed for the folded peptide, in this case the retention time is slightly increased (23.20 min) due to the removal of the two hydrophilic Acm groups. The major peaks in the HPLC chromatograms of crude partially folded peptide **2** and fully folded lt5d were collected and analyzed using HR-ESI(+)-MS. Fig. 4 shows the molecular ion peaks



Scheme 2 Lt5d synthesis using orthogonal cysteine protection (Method 2).



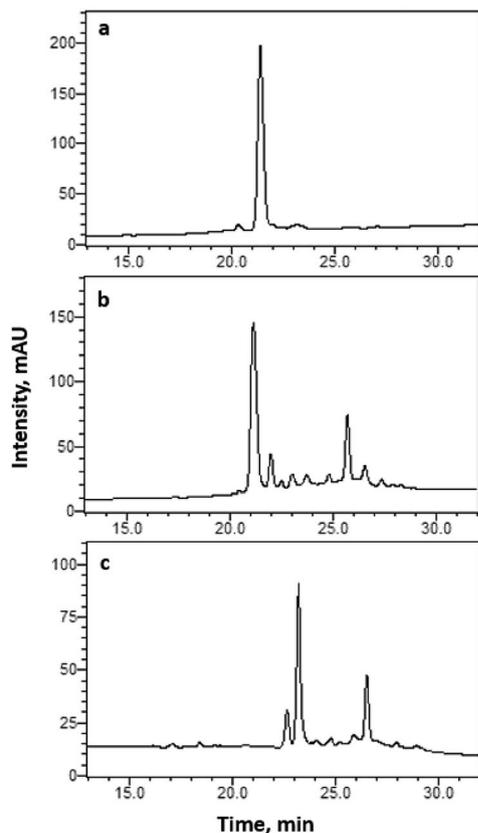


Fig. 3 RP-HPLC chromatograms of crude (a) 1, (b) 2, and (c) It5d with $t_R = 21.39$, 21.14 and 23.20 min, respectively. Analytical C_{18} column was used to profile the sample with a linear gradient from 15% to 45% solution B (90% MeCN in H_2O with 0.1% TFA) for 35 min at 1 mL min^{-1} flow rate. Absorbance was monitored at 220 nm.

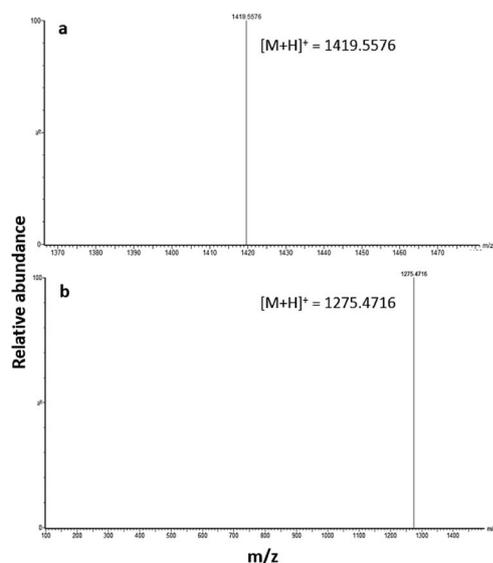


Fig. 4 HR-ESI(+) mass spectra of pure (a) 2 at 1419.5576, and (b) folded It5d, at 1275.4716. Calculated monoisotopic mass of 2 and It5d is 1418.6278 and 1274.4916 Da, respectively.

observed at $m/z = 1419.5576$ for 2 (one disulphide bond, *i.e.*, Cys2–Cys9) and $m/z = 1275.4716$ for It5d (two disulphide bonds, *i.e.*, Cys2–Cys9 and Cys3–Cys10). The mass difference of 144 Da between these compounds corresponds to the removal of the two Ac groups.

In order to confirm the structural similarity of the peptides obtained from Method 1 and Method 2, the purified peptide products from the two methods were co-injected and analyzed by HPLC. The chromatograms in Fig. 5 show that the two compounds exhibit the same retention time, confirming that the synthesized peptides have the same folding configuration. Since Method 2 guarantees the controlled, sequential formation of disulphide bonds Cys2–Cys9 and Cys3–Cys10, its co-elution with the glutathione-folded product from Method 1 confirms that the same disulphide linkages were formed. Furthermore, co-injection of the two peptide products in HR-ESI(+)-MS also yielded the same molecular ion peaks (Fig. S1†).

Finally, both peptides were also characterized by 1H NMR spectroscopy (500 MHz) in $DMSO-d_6$, and the similarities in chemical shifts, peak shape, and integration further confirm that the peptides obtained from both methods are identical

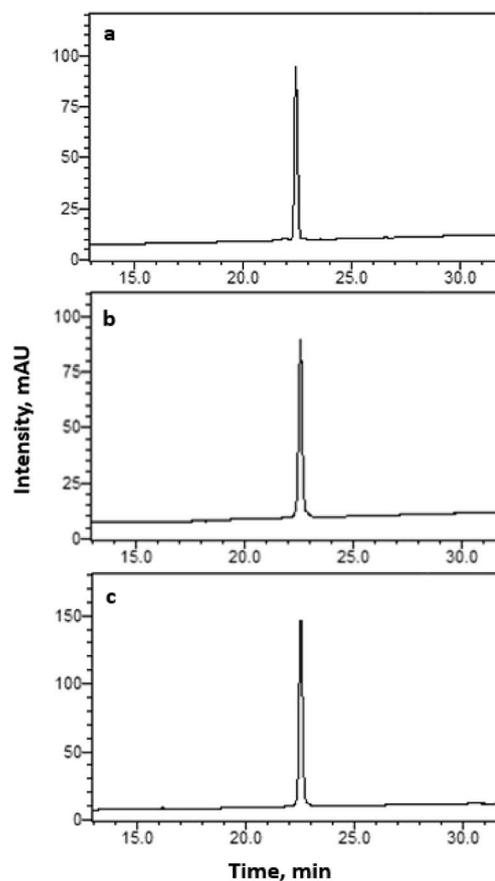


Fig. 5 RP-HPLC chromatograms of It5d synthesized using (a) Method 1, glutathione folding ($t_R = 22.43$ min), (b) Method 2, orthogonal cysteine protection ($t_R = 22.53$ min), and their (c) co-injection ($t_R = 22.53$ min). Analytical C_{18} column was used to profile the samples with a linear gradient from 15% to 45% solution B (90% MeCN in H_2O with 0.1% TFA) for 35 min at 1 mL min^{-1} flow rate. Absorbance was monitored at 220 nm.



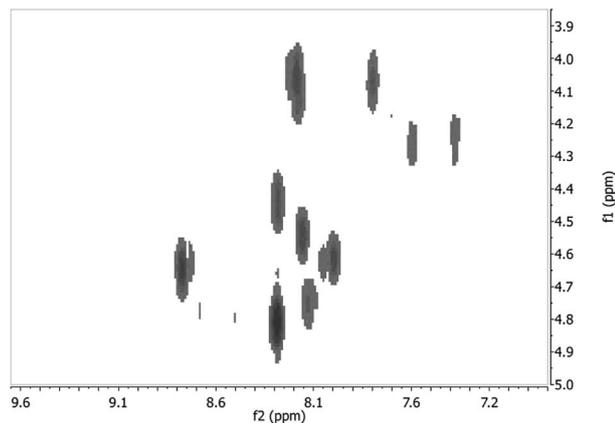


Fig. 6 Fingerprint region of the H–H COSY NMR spectrum of It5d obtained from Method 2 (500 MHz, DMSO- d_6). The ten (10) cross-peaks observed correspond to the correlations of NH protons with ten (10) of the twelve (12) H_α protons of the It5d amino acid sequence. As Pro has no amide proton, correlations for the two (2) Pro residues in the sequence are absent.

(Fig. S2†). The ^1H NMR spectrum of the peptide obtained from Method 2 exhibited peaks of better resolution, therefore was characterized further using 2D NMR techniques. In particular, H–H COSY spectroscopy permitted the identification of ten (10) amino acids through the cross-peaks observed in the fingerprint region of the NMR spectrum (Fig. 6).¹⁸ These correspond to the ten (10) amino acids in the sequence of It5d, with the exception of the two (2) Pro residues. A more detailed assignment of individual spin systems to these cross-peaks can be derived from the TOCSY spectrum (Fig. S3†). For instance, the NMR signals arising from Leu were readily identifiable based on the cross-peaks of amide protons at 8.19 and 7.36 ppm with the resonance peaks of aliphatic protons at 0.84, 1.45, and 1.54 ppm. Pro was identified based on the cross-peaks between 1.80 and 3.50 ppm, and the absence of cross-peaks between H_α protons in the ~4.10 to 4.80 ppm region with amide protons in ~6.80 to 8.77 region. The four (4) Cys residues are identifiable based on the signature cross-peaks of NH, H_α and H_β signals, although the sequence-specific resonance of each individual Cys was indistinguishable. The results of these NMR analyses provide further structural confirmation of the target peptide It5d.

Conclusions

The successful synthesis of It5d is reported for the first time. It has been shown that the two strategies described above, *i.e.*, glutathione-assisted oxidative folding (Method 1) and orthogonal cysteine protection (Method 2), yield the same product as confirmed by HPLC, MS, and NMR data. The capability of producing large amounts of purified peptide is particularly necessary in order to conduct a comprehensive *in vitro* and *in vivo* pharmacological assessment of the peptide. For instance, in order to determine its specific molecular target, It5d can be assessed by different bioactivity assays, such as electrophysiology using cells expressing a specific type of TTX-sensitive Na

channel isoform (*e.g.* $\text{Na}_v1.1$ –4, $\text{Na}_v1.6$ and $\text{Na}_v1.7$).^{2,19,20} Voltage-gated sodium channel isoforms $\text{Na}_v1.3$ and $\text{Na}_v1.7$ are among those identified to be involved in neuropathic pain.²¹ As It5d has been previously reported to have high inhibitory activity against TTX-sensitive Na channels ($\text{IC}_{50} = 156.16$ nM),¹ it is a peptidic drug candidate worth pursuing.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was supported by the Department of Science and Technology – Philippine Council for Health Research and Development (DOST PCHRD, FP140015 “Project 2: Anti-Pain and Anti-Neurodegeneration Drug Candidates: Discovery and Development” under the “Discovery and Development of Health Products (DDHP) Marine Component”). Special thanks to Dessa Camille Batoctoy, Vincenzo Paolo Torreno and Ramoncito Luis de Boda for generating the ESI-MS data.

Notes and references

- J. Liu, Q. Wu, C. Pi, Y. Zhou, M. Zhou, L. Wang, S. Chen and A. Xu, *Peptides*, 2007, **28**, 2313–2319.
- K. B. Akondi, M. Muttenthaler, S. Dutertre, Q. Kaas, D. J. Craik, R. J. Lewis and P. F. Alewood, *Chem. Rev.*, 2014, **114**, 5815–5847.
- P. Anand, A. O’Neil, E. Lin, T. Douglas and M. Holford, *Sci. Rep.*, 2015, **5**, 12497.
- M. Sanford, *CNS Drugs*, 2013, **27**, 989–1002.
- D. P. Wermeling, *Pharmacotherapy*, 2005, **25**, 1084–1094.
- S. V. Phan and J. M. Waldfoegel, *General Hospital Psychiatry*, 2015, **37**, 97.e11–97.e12.
- WoRMS Editorial Board, *World Register of Marine Species*, available from <http://www.marinespecies.org> at VLIZ, 2017, DOI: 10.14284/170.
- Q. Kaas, R. Yu, A. H. Jin, S. Dutertre and D. J. Craik, *Nucleic Acids Res.*, 2012, **40**, D325–D330.
- Q. Kaas, J. C. Westermann, R. Halai, C. K. Wang and D. J. Craik, *Bioinformatics*, 2008, **24**, 445–446.
- K. K. Khoo, K. Gupta, B. R. Green, M.-M. Zhang, M. Watkins, B. M. Olivera, P. Balaram, D. Yoshikami, G. Bulaj and R. S. Norton, *Biochemistry*, 2012, **51**, 9826–9835.
- M. Okumura, M. Saiki, H. Yamaguchi and Y. Hidaka, *FEBS J.*, 2011, **278**, 1137–1144.
- S. Chakravarthi, C. E. Jessop and N. J. Bulleid, *EMBO Rep.*, 2006, **7**, 271–275.
- S. A. Esperante, G. Covalada, S. A. Trejo, S. Bronsoms, F. X. Aviles and S. Ventura, *Sci. Rep.*, 2017, **7**, 5457.
- G. Bulaj, O. Buczek, I. Goodsell, E. C. Jimenez, J. Kranski, J. S. Nielsen, J. E. Garrett and B. M. Olivera, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 14562–14568.
- E. Fuller, B. R. Green, P. Catlin, O. Buczek, J. S. Nielsen, B. M. Olivera and G. Bulaj, *FEBS J.*, 2005, **272**, 1727–1738.



Paper

- 16 A. Cuthbertson and B. Indrevoll, *Org. Lett.*, 2003, 5, 2955–2957.
- 17 E. Klüver, S. Schulz-Maronde, S. Scheid, B. Meyer, W.-G. Forssmann and K. Adermann, *Biochemistry*, 2005, 44, 9804–9816.
- 18 C. Redfield and C. M. Dobson, *Biochemistry*, 1988, 27, 122–136.
- 19 J. R. Prashanth, N. Hasaballah and I. Vetter, *Neuropharmacology*, 2017, 127, 4–19.
- 20 C. H. Lee and P. C. Ruben, *Channels*, 2008, 2(6), 407–412.
- 21 S. D. Dib-Hajj, J. A. Black and S. G. Waxman, *Pain Med.*, 2009, 10, 1260–1269.

