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Hydrazine hydrate facilitates efficient synthesis of cyclodipeptides for antibacterial screening

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We report a facile and efficient strategy for synthesizing cyclodipeptides through hydrazine hydrate-mediated cyclization of dipeptide esters under exceptionally mild conditions (room temperature, ethanol). This operationally simple protocol enables the gram-scale construction of 33 diverse cyclodipeptides from Boc-protected precursors in good yields (53–91%), without requiring heating, column chromatography, or incurring racemization. Notably, antibacterial screening revealed that four newly reported cyclodipeptides exhibit activity against *E. coli*, underscoring the utility of this method for generating bioactive molecules.

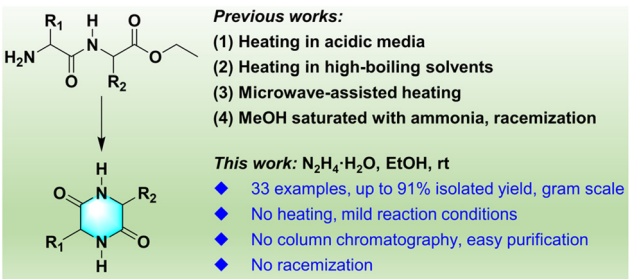
Introduction

Cyclodipeptides, commonly referred to as 2,5-diketopiperazines (DKPs), have attracted significant attention due to their diverse biological activities and drug discovery potential, including anticancer, antibacterial, antifungal, antiviral, and antioxidant properties, as well as roles in intercellular signaling and the regulation of energy metabolism.[1–7] Furthermore, their remarkable self-assembly characteristics facilitate the formation of functional nanomaterials.[8–11] Consequently, the efficient preparation of cyclodipeptides is crucial for advancing research and development in their potential applications.

Compared with extraction from fungi and bacteria, as well as biosynthetic and solid-phase synthesis methods,[1,4] solution-phase synthesis provides an efficient strategy for large-scale production of cyclodipeptides. Among various solution-phase approaches, dipeptide ester cyclization stands out as a particularly prevalent method.[2] Traditional protocols involve thermal treatment in acidic media[12] or high-boiling solvents,[13] but microwave-assisted heating[14] has emerged as a versatile and efficient alternative (Scheme 1). This modern technique demonstrates broad applicability across diverse amino acid sequences. Heating is typically essential in these reactions, as elevated temperatures enhance the conformational flexibility of the peptide chain. This increased mobility allows the molecular chain to fold and coil more freely, facilitating the formation of *cis*-amide bonds. By bringing the reactive amino and ester groups into close proximity, thermal activation promotes intramolecular cyclization. However, these demanding reaction conditions often necessitate specialized equipment. Moreover, racemization remains a potential issue under certain harsh circumstances,[7] posing challenges to the synthesis of optically pure cyclodipeptides.

Subjecting dipeptide esters to a basic environment, such as an excess of ammonia in the Fischer method (Scheme 1),[15,16] provides a straightforward approach for synthesizing cyclodipeptides. However, this method has been reported to result in varying degrees of racemization. It is suggested that this racemization occurs during the exposure of unreacted dipeptide esters to basic ammonia.[17] Consequently, identifying a suitable weak base that facilitates cyclization without inducing racemization of dipeptide esters is essential for the efficient synthesis of cyclodipeptides.

Hydrazine hydrate ($N_2H_4{\cdot}H_2O$) is the hydrated form of hydrazine (N_2H_4), which has significant applications in indus-



Scheme 1 Reaction conditions for dipeptide ester cyclization to form cyclodipeptides in previous works and in this work.

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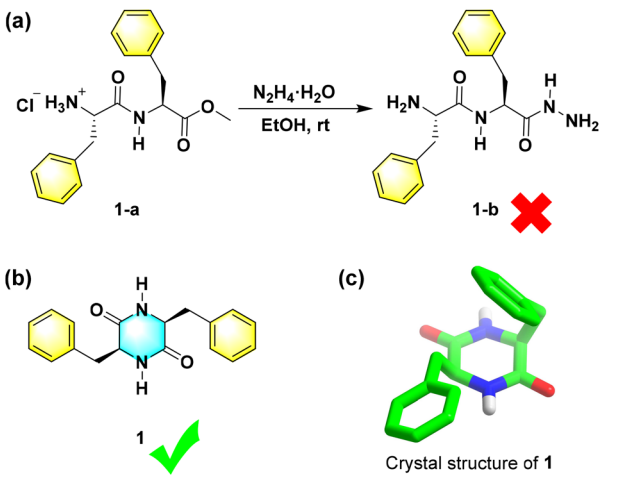
try and research, especially in the synthesis of pharmaceuticals and agrochemicals. In this regard, $N_2H_4{\cdot}H_2O$ has been used in the production of peptide-related compounds. For example, in solid-phase peptide synthesis, as a base, $N_2H_4{\cdot}H_2O$ is an effective reagent for the removal of the Fmoc group.[18] Additionally, we have used $N_2H_4{\cdot}H_2O$ as a reactant in the synthesis of short peptide-based hydrazides, which can then produce peptidomimetics.[19–25] These applications of $N_2H_4{\cdot}H_2O$ suggest that exposure of peptides to its basic environment does not induce racemization, a feature attributable to its weak basicity. Specifically, the basicity of $N_2H_4{\cdot}H_2O$ is weaker than that of ammonia. This is because each nitrogen atom in hydrazine bears an electron-withdrawing amino group, which reduces the electron density on the nitrogen lone pairs, rendering them less available for protonation compared to those in ammonia. Consequently, this diminished basicity lowers the ability of hydrazine to abstract α-protons from the peptide backbone, thereby minimizing the risk of racemization. On this basis, we anticipated that $N_2H_4{\cdot}H_2O$ could serve as an efficient weak base for cyclodipeptide synthesis, and our experimental results confirmed its effectiveness in this role (Scheme 1).

Results and discussion

We initiated our study by using commercial diphenylalanine methyl ester hydrochloride (**1-a**) as the starting substrate. This substrate was reacted with $N_2H_4{\cdot}H_2O$ (85% aqueous solution) in EtOH at room temperature, as depicted in Scheme 2a. Our previous research had successfully employed similar reaction conditions to synthesize short peptide-based hydrazides, utilizing N-protected peptide esters as substrates.[19–25] However, the substantial precipitate formed in this current reaction was found not to be the expected diphenylalanine-based hydrazide **1-b**. Analysis of the NMR spectra (Fig. S1 and S2) revealed that the two phenylalanine residues within the product exhibited identical chemical environments, indicating a high degree of structural symmetry. This strongly suggested that the product was cyclic diphenylalanine **1**. This hypothesis was subsequently corroborated by HRMS analysis (Fig. S3) and definitively confirmed by single-crystal X-ray diffraction (Scheme 2c and Table S1).



Scheme 2 (a) The reaction of diphenylalanine methyl ester hydrochloride (**1-a**) with hydrazine hydrate ($N_2H_4{\cdot}H_2O$) in EtOH under room temperature, yielding not anticipated diphenylalanine-based hydrazide (**1-b**). (b) Cyclic diphenylalanine **1** was identified to be the product. (c) Crystal structure of **1**. For clarity, −CH protons are omitted. This structure is consistent with the previously reported crystal structure of **1**.[8,11]

Based on this finding, we hypothesized that $N_2H_4{\cdot}H_2O$ plays a critical role in facilitating the cyclization of N-unprotected dipeptide esters into cyclodipeptides. Reaction conditions were subsequently optimized using 36.2 mg of substrate **1-a** in 1.0 mL of solvent (0.1 M). Optimization revealed that 2.0 equiv. of $N_2H_4{\cdot}H_2O$ were optimal for achieving a high yield of product **1** (Table S2). We propose that one equiv. neutralizes the hydrochloride salt of substrate **1-a**, while the second equiv. serves as a catalyst to promote the cyclization. Furthermore, mild conditions, room temperature (25 °C) and a 12-hour reaction time, proved sufficient for high-yield production of **1** (Tables S3 and S4).

Subsequently, we examined the effect of solvent on the reaction. Compared to apolar solvents such as toluene and *n*-hexane, polar solvents, including alcohols, water, and DMF, afforded significantly higher yields, exceeding 75% (Table 1). In particular, alcohols (MeOH, EtOH, *n*-PrOH, and *n*-BuOH) and water proved highly favorable for the formation of product **1**, yielding over 90%. This enhancement can be attributed to the ability of polar solvents to increase the basicity of $N_2H_4{\cdot}H_2O$, thereby activating the reaction between the amino and ester groups and facilitating cyclization. In contrast, solvents such as MeCN and EtOAc tend to react with $N_2H_4{\cdot}H_2O$,[26] which suppresses the formation of **1**. Considering its high yield (97%), good solubility for various substrates, ease of removal, and environmentally benign nature, EtOH was selected as the solvent for all subsequent reactions.

Table 1 Yields of **1** obtained in the cyclization reactions using 36.2 mg of **1-a** in different solvents (1.0 mL) stirred at 25 °C for 12 h in the presence of 2.0 equiv. of $N_2H_4{\cdot}H_2O$

Entry	Solvent	Yield[a]/%
1	MeOH	95
2	EtOH	97
3	*n*-PrOH	97
4	*n*-BuOH	97
5	H_2O	94
6	DMF	75
7	MeCN	52
8	EtOAc	49
9	THF	49
10	CH_2Cl_2	42
11	$CHCl_3$	51
12	Toluene	42
13	*n*-Hexane	45
14	Cyclohexane	48

[a] Reaction yields determined by ^{1}H NMR spectra.

We further investigated the influence of base properties on the formation of cyclodipeptide **1**. In addition to $N_2H_4{\cdot}H_2O$, several common bases were systematically evaluated. Analogues of $N_2H_4{\cdot}H_2O$, namely NH_2OH and $NH_3{\cdot}H_2O$, were also found to promote the conversion of **1-a** to **1**, with both $N_2H_4{\cdot}H_2O$ and NH_2OH affording yields exceeding 90%. In contrast, although stronger bases such as diethylamine, triethylamine, and diisopropylethylamine possess sufficient basicity to enable amide bond formation *via* amino-ester coupling, they afforded product **1** with low efficiency (Table 2). This outcome can be plausibly explained by their inability to participate in hydrogen bonding, which appears essential for stabilizing the putative *cis*-amide conformation in the dipeptide intermediate. Meanwhile, aniline, being too weakly basic, failed to promote the amide bond formation. These findings indicate that $N_2H_4{\cdot}H_2O$ not only provides a suitable basic environment to facilitate amide coupling, but also stabilizes the otherwise disfavored *cis*-amide conformation through hydrogen-bonding interactions with the peptide backbone. This preorganization facilitates intramolecular aminolysis under basic conditions, leading to diketopiperazine formation *via* a low-energy cyclic transition state involving proton shuttling (Scheme S1).[27]

Given that the stronger basicity of $NH_3{\cdot}H_2O$ could lead to racemization,[15,16] $N_2H_4{\cdot}H_2O$ and NH_2OH are recommended as suitable weak bases for the straightforward synthesis of cyclodipeptides from linear dipeptide esters. We initially selected $N_2H_4{\cdot}H_2O$ due to its lower cost. Under optimized conditions (EtOH, rt, 12 h, 2.0 equiv. of $N_2H_4{\cdot}H_2O$), we successfully obtained 148 mg of pure **1** from 200 mg of **1-a** as the substrate. This was achieved through a simple washing of the precipitate with ethyl ether, resulting in an isolated yield of 91%.

To investigate the generality of this method, we designed a three-step synthetic procedure for the synthesis of diverse cyclodipeptides (Scheme 3). (i) The coupling of an *N*-Boc-protected amino acid with an amino acid ethyl ester using 1-hydroxybenzotriazole (HOBT), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and triethylamine (Et_3N) produces *N*-Boc-protected dipeptide ethyl esters. (ii) The Boc group was removed using trifluoroacetic acid, yielding the dipeptide ethyl esters as their corresponding trifluoroacetate salts. (iii) The dipeptide ethyl esters were cyclized in EtOH in the presence of $N_2H_4{\cdot}H_2O$ at 25 °C for 12 h to form cyclodipeptides. The initial amount of substrate was 10 mmol, and the final cyclization reaction was conducted in 30 mL ethanol. To address the concern of acidity due to residual trifluoroacetic acid from step (ii), 11 equiv. of $N_2H_4{\cdot}H_2O$ were introduced during the cyclization (Scheme S2). Thermal treatment is typically essential for the synthesis of cyclodipeptides,[12–14] but our method presents an alternative approach that circumvents the need for such heating. This allows for the synthesis process to be gentler, minimizing the likelihood of racemization, side reactions, or degradation of sensitive reactants.

Table 2 Yields of **1** obtained in the cyclization reactions using 36.2 mg of **1-a** in EtOH (1.0 mL) stirred at 25 °C for 12 h in the presence of 2.0 equiv. of different bases

Entry	Base	Yield[a]/%
1	—	0
2	$N_2H_4{\cdot}H_2O$	97
3	NH_2OH	93
4	$NH_3{\cdot}H_2O$	83
5	Diethylamine	48
6	Triethylamine	41
7	Diisopropylethylamine	19
8	Aniline	0

[a] Reaction yields determined by ^{1}H NMR spectra.

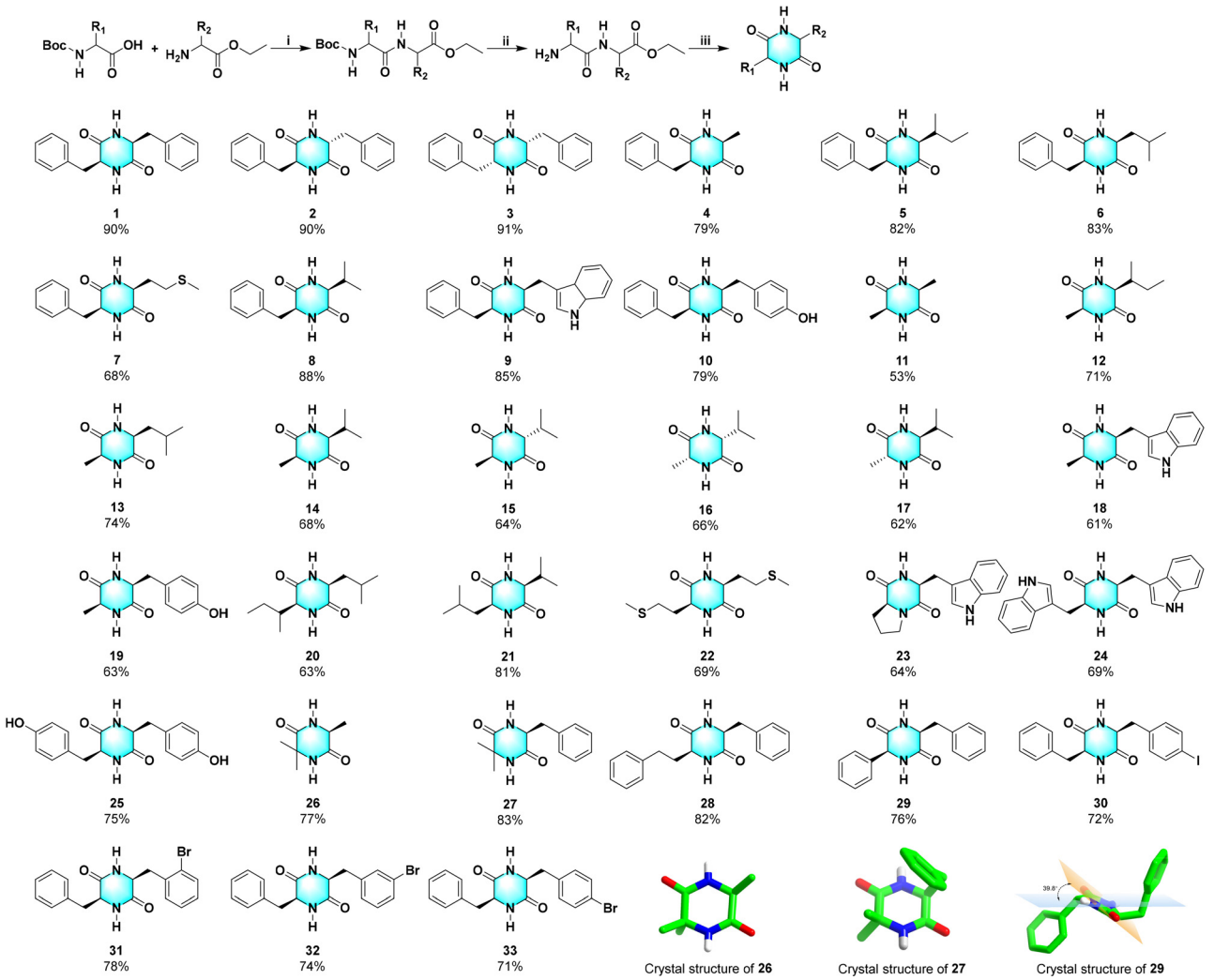
Based on this procedure, we synthesized 33 cyclodipeptides with overall yields ranging from 53% to 91%, all produced on a gram scale (Scheme 3). The yield is influenced by the nature of the amino acid residues. For instance, the presence of the bulky phenylalanine residue facilitates crystallization and purification, resulting in a notably high overall yield of 90% for **1**. Conversely, the small, amphiphilic alanine residue negatively impacts purification efficiency, leading to a lower yield of 53% for **11**.

Moreover, modifications to the amino acid configuration did not substantially affect cyclization efficiency or purification feasibility, as evidenced by the comparable overall yields of stereoisomers in cyclic diphenylalanine (**1**/**2**/**3**) and cyclic alanine-valine (**14**/**15**/**16**/**17**) systems. Chiral HPLC analyses of the synthesized stereoisomers (Fig. S4 and S5) confirmed the absence of racemization during cyclization, demonstrating that $N_2H_4{\cdot}H_2O$ acts as an effective mild base for cyclodipeptide synthesis with complete stereochemical retention. This outcome is attributed to the moderate basicity of $N_2H_4{\cdot}H_2O$, which minimizes racemization pathways while enabling efficient cyclization.

Non-canonical amino acids, including 2-aminoisobutyric acid, homophenylalanine, and 2-phenylglycine, were successfully employed to synthesize cyclodipeptides **26–29** in good overall yields (76–83%; Scheme 3). Crystals of **26**, **27**, and **29** suitable for X-ray diffraction were obtained by slow evaporation (Tables S5–S7). Their crystal structures unambiguously confirmed the formation of the cyclodipeptide framework (Scheme 3). A pronounced conformational difference was observed in the diketopiperazine ring, which is more twisted in **29** (39.8°) than in **26** (18.9°) and **27** (6.36°). The substantial distortion in **29** results from the steric hindrance imposed by the directly fused bulky benzene ring.

Halide-substituted phenylalanines were also employed in cyclodipeptide synthesis, affording compounds **30–33** with comparable overall yields (71–78%, Scheme 3). This observation suggests that the substitution position of halides on the aromatic ring does not significantly impact the reaction efficiency. However, compared to the unsubstituted analogue **1** (90% yield), the diminished yields of **30–33** are plausibly attributed to increased losses during purification arising from the enhanced polarity introduced by halide substituents.

Absorption and circular dichroism (CD) spectra of all 33 synthesized cyclodipeptides were recorded in MeCN (Fig. S6–



Scheme 3 Developed three-step synthetic procedure for cyclodipeptides using *N*-Boc-protected amino acids and amino acid ethyl ester as initial substrates, and structures of the synthesized cyclodipeptides and their overall isolated yields. Crystal structures of **26**, **27**, and **29** are shown, wherein –CH protons are omitted for clarity. (i) HOBt/EDCI/Et_3N, $CHCl_3$; (ii) CF_3COOH, CH_2Cl_2; (iii) $N_2H_4{\cdot}H_2O$, EtOH, rt.

S9). With the exception of the CD-silent mesomer **2**, all compounds exhibited distinct CD signals, and the spectral profiles were found to depend on the constituent amino acid residues. These results confirm that chirality was preserved throughout the synthetic processes.

The successful synthesis of 33 cyclodipeptides underscores the versatility of $N_2H_4{\cdot}H_2O$ in facilitating the cyclization of dipeptide esters, which proceeds under mild conditions (EtOH, rt) without requiring column chromatography or inducing racemization (Scheme 1). This methodology offers notable advantages including high yields, operational simplicity, and excellent stereochemical fidelity.

Given the significant biological activities of cyclodipeptides, particularly their antibacterial potential,[28–33] we evaluated the antibacterial properties of the synthesized 33 cyclodipeptides. Initially, an agar well diffusion assay was used to assess their activity against *Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923), and *Pseudomonas aeruginosa* (ATCC27853) by measuring inhibition zone diameters. Among these, four compounds showed antibacterial activity against *E. coli* (Fig. 1a), all of which were newly reported: cyclo(-L-alanyl-L-

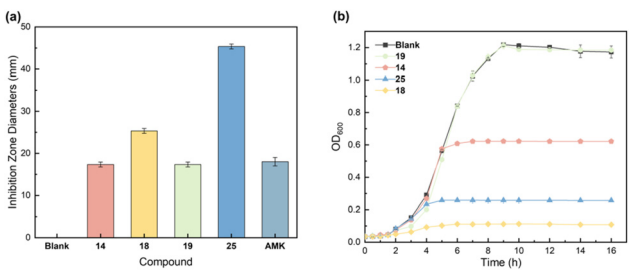


Fig. 1 (a) Inhibition zone diameters of compounds **14**, **18**, **19**, **25**, and amikacin (AMK) against *E. coli*. Concentrations of **14**, **18**, **19**, and **25** were 4 mg mL^{-1}, and positive control AMK was 10 μg mL^{-1}. (b) 16-hour growth inhibition curves of **14**, **18**, **19**, and **25** against *E. coli* at a concentration of 125 μg mL^{-1}.

valine) (**14**), cyclo(-L-alanyl-L-tryptophan) (**18**), cyclo(-L-alanyl-L-tyrosine) (**19**), and cyclo(-L-tyrosyl-L-tyrosine) (**25**). The minimum inhibitory concentrations (MICs) of these four cyclodipeptides against *E. coli* were further determined by broth microdilution in 96-well plates, with MIC values of 250 μg mL^{-1} for **14**, 125 μg mL^{-1} for **18**, 1000 μg mL^{-1} for **19**, and 167.5 μg mL^{-1} for **25**. The 16-hour growth inhibition curves (Fig. 1b) showed that at a uniform concentration of 125 μg mL^{-1}, the inhibition rates reached 41% for **14**, 91% for **18**, 0% for **19**, and 77% for **25**. These findings establish a solid basis for subsequent research, including combination therapy studies, *in vivo* antibacterial assessment in animal models, and mechanistic investigations.

Conclusions

In summary, we have developed a mild and efficient method for synthesizing cyclodipeptides through the cyclization of dipeptide esters using $N_2H_4{\cdot}H_2O$ in EtOH at room temperature. Starting from Boc-protected amino acids and amino acid esters, a series of 33 cyclodipeptides were prepared *via* a three-step route, with $N_2H_4{\cdot}H_2O$ employed as the key cyclization agent in the final step, affording isolated yields of 53% to 91%. This strategy not only provides efficient access to structurally diverse cyclodipeptides but also holds promise for the synthesis of macrocyclic oligoamides,[34,35] indicating its potential utility in drug discovery. Importantly, antibacterial evaluation revealed that four of the obtained cyclodipeptides exhibited activity against *E. coli*, all of which have not been previously reported for antibacterial activity, a finding that directs our ongoing research into their antibacterial properties.

Author contributions

Z. L. and C. W. performed the synthesis and conducted data analysis. Y. D. carried out the antibacterial assays and analyzed the data. Y. W., J. T. and X. G. assisted with the experiments and provided technical support and helpful discussions. Y.-B. J. and X. Y. conceived and supervised the project and revised the manuscript. The manuscript was written through contributions of all authors.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d6ob00499g.

CCDC 2416472 (**27**), 2491940 (**26**) and 2491941 (**29**) contain the supplementary crystallographic data for this paper.[36a–c]

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