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Loops on loops: generation of complex scaffolded peptides presenting multiple cyclic fragments

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Scaffolded peptides presenting two different cyclic peptide fragments through a cyclic peptidomimetic scaffold in a site-selective fashion, were generated by stepwise solid phase synthesis and fragment condensation in parallel, demonstrating that either strategy is adequate to generate complex scaffolded peptides.

Introduction

Scaffolded peptides, in which protein-derived peptide fragments are presented in a non-linear, sequentially discontinuous fashion, are promising tools for the mimicry of discontinuous protein binding sites.[1] In such scaffolded peptides, protein fragments making up the binding sites are presented through a molecular scaffold, enabling the site-selective attachment of individual, different fragments to defined sites of the scaffold. Various types of molecules have been proposed as scaffolds, including steroids,[2] triazacyclophanes,[3] cyclic[4] and bicyclic peptides,[5] as well as so-called tweezer molecules.[6]

The site-selective attachment of up to three different linear fragments to a molecular scaffold can be achieved in high purity through solid-phase synthesis of the scaffold, followed by successive, stepwise assembly of the three fragments at defined sites of the support-bound scaffold.[7] The discontinuous binding sites of many proteins, however, contain at least one fragment having a hair pin or other loop-like structures within the protein, which should be presented as a cyclic peptide fragment in scaffolded peptides. The generation of scaffolded peptides presenting cyclic peptide fragments, however, continues to be a challenge in peptide synthesis, as the stepwise synthesis of such molecules involves repeated cyclization reactions on the solid support. Convergent synthesis strategies,[8] such as solid-phase fragment condensation, on the other hand, which were developed in order to improve the yield and quality of chemically synthesized large peptides and proteins, require the separate synthesis of fully protected peptide fragments using different linker strategies. The protected peptide fragments are generated as C-terminal peptide acids using mildly cleavable linkers, such as the 2-chlorotrityl,[9,10] Sasrin™,[11] or allyl-based linkers,[12] enabling cleavage of peptides without concurrent removal of N-terminal and side chain protecting groups, and subsequently coupled to another peptide fragment on the solid support. Such convergent synthesis schemes have also been used to couple linear protected peptide fragments to support-bound scaffold molecules.[13,14] For the generation of highly complex scaffolded peptide libraries,[5] convergent synthesis strategies offer the additional benefit of cross-combining scaffolds and peptide fragment mixtures in a combinatorial fashion, thus enabling the generation of multiple, diverse libraries from relatively small sets of scaffolds and peptide fragment mixtures.

The goal of this study was to establish synthesis strategies for the generation of complex scaffolded peptides, in which different cyclic peptide fragments are attached site-selectively to defined sites of a cyclic peptidomimetic scaffold, based on stepwise solid-phase synthesis, and solid-phase fragment condensation, respectively.

Results and discussion

Our strategy for the generation of scaffolded peptides as synthetic mimetics of discontinuous protein binding sites is based on the use of cyclic peptides having varying ring sizes as scaffold molecules, to which peptide fragments are attached in a site-selective fashion. We have previously shown that scaffolds with ring sizes ranging from 13 to 29, covering a wide range of conformational flexibility, can be generated by incorporating into the scaffolds spacer amino acids with different backbone lengths (–NH–$(CH_2)_n$–CO–, n = 1–5).[7] In this study, we used two different scaffolds with ring sizes of 20 (**1**) and 22 (**2**) atoms, respectively, which were generated using GABA (–NH–$(CH_2)_3$–CO–) as spacer amino acid (Fig. 1).

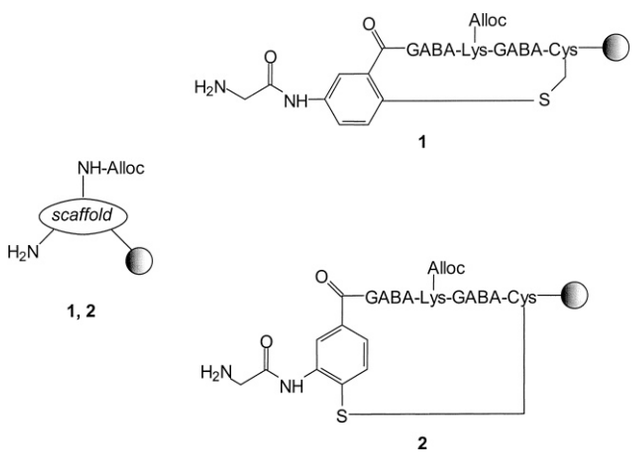

Fig. 1 General (left) and specific (right) structures of **1** and **2**.

In order to evaluate and compare stepwise and convergent synthesis methods for the attachment of cyclic peptide fragments to defined sites of scaffolds **1** and **2**, scaffolded peptides **7** and **8** (Fig. 2), each presenting two different model cyclic peptide fragments, were synthesized following either protocol.

For the convergent strategy (Scheme 1, route A), cyclic peptide fragments **3** and **6** were first separately synthesized as fully protected C-terminal acids on the 2-chlorotrityl linker. The two amino groups of the scaffolds, serving as selectively addressable sites for attaching peptide fragments, were either Alloc-protected, or generated through reduction of an aromatic nitro group, followed by acylation of the resulting aromatic amino group with glycine.[7] The first cyclic peptide fragment, *cyclo*[Succ-YAFGYPSK]G, containing the L-Ala analog of the opioid-like peptide dermorphin from frog skin,[15] was attached to (fragment condensation, Scheme 1, route A), or assembled

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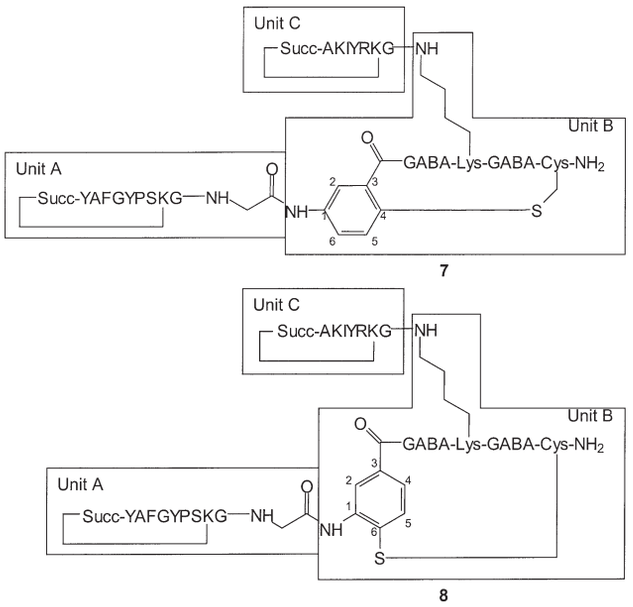


Fig. 2 Structures of **7** and **8**. Boxed units and numbered residues refer to NMR analysis (Tables 1 and 2).

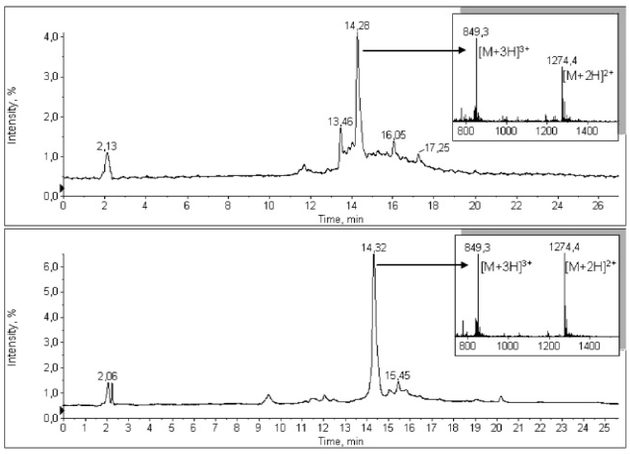


Fig. 3 HPLC chromatograms and mass spectra of crude **7** generated through fragment condensation (top; the peak with a retention time of 13.46 min represents a side product resulting from incomplete coupling of **6**), and stepwise synthesis (bottom). See experimental section for conditions.

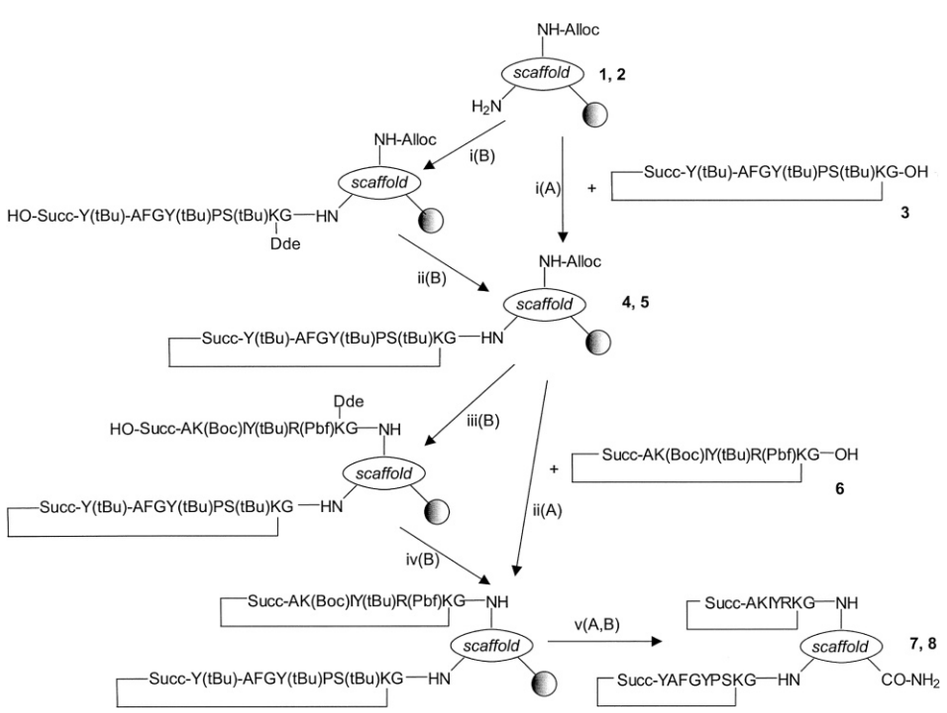
at (stepwise synthesis, Scheme 1, route B), the glycine amino group of the scaffolds, affording scaffolded peptides **4** and **5**. The second cyclic peptide fragment, *cyclo*[Succ-AKIYRK]G, containing the core sequence of a trypsin inhibitor derived from a synthetic peptide library,[16] was attached to (fragment condensation), or assembled at (stepwise synthesis), the lysine side chain of the scaffolds after removal of the Alloc group, affording scaffolded peptides **7** and **8**, which were cleaved from the solid support and analyzed by HPLC-MS.

All crude peptides were of high purity and had the correct molecular mass (Figs. 3 and 4). The purities of the products obtained by stepwise assembly were still somewhat higher than those of the fragment condensation products, in which incomplete coupling of **6**, presumably due to steric hindrance, resulted in the formation of side products.

The synthesis products obtained following the two different protocols had identical mass spectra (Figs. 3 and 4), and co-eluted after co-injection on the HPLC column (Fig. 5), demonstrating that the products from both syntheses are identical. In a control experiment, **7** and **8**, which are very similar, were clearly separated after co-injection (data not shown). For further characterization by high-resolution ESI-MS and MALDI-MS, as well as ^{1}H NMR spectroscopy, **7** and **8** were purified by preparative HPLC.

Characterization of 7 and 8 by ^{1}H NMR spectroscopy

The identification of the various spin systems in **7** and **8** (amino acid residues, succinyl moiety, GABA and aromatic systems) was readily determined from the two-dimensional TOCSY and NOESY spectra, and is shown in Tables 1 and 2. The NOESY spectra afforded unambiguous sequential assignment of the amino acid residues in units A, B and C (see Fig. 2) from cross peaks corresponding to (i, i + 1) NHNH and (i, i + 1) αHNH



Scheme 1 Generation of **7** and **8** through solid-phase fragment condensation (route A), and stepwise solid-phase synthesis (route B). i(A): Coupling of **3**, ii(A): Alloc-removal, coupling of **6**. i(B): Stepwise assembly of linear peptide sequence. ii(B), iv(B): Dde-removal, cyclization. iii(B): Alloc-removal, stepwise assembly of linear peptide sequence. v(A,B): Cleavage from solid support with concurrent deprotection of side chains. Succ: –CO–$(CH_2)_2$–CO–. See experimental section for details.

Table 1 ^{1}H chemical shifts of **8** at 300 K in 50% aqueous CD_3CN

	HN	Hα	Hβ	Hγ	Hδ	Hε	Others
Unit A							
Succ		2.29	2.39				
Tyr-1	7.80	4.30	2.90, 2.78				H-3,5: 6.68, H-2,6: 6.99
Ala-2	7.93	4.07	1.08				
Phe-3	7.54	4.44	3.05, 2.93				H-2,6: 7.12, H-3,5: 7.23, H-4: 7.17
Gly-4	7.86	3.81, 3.34					
Tyr-5	7.71	4.63	2.96, 2.84				H-3,5: 6.68, H-2,6: 6.99
Pro-6[b]	—	4.22	2.10, 1.82	1.90, 1.82	3.66, 3.52		
Ser-7	7.65	4.44	3.78, 3.71				
Gly-9	7.98	3.89					
Gly-10	8.04	3.99					
Unit B							
Arom[a]	H-2: 7.82, H-4: 7.45, H-5: 7.51, NH: 9.19						
GABA	8.23	3.34, 3.28	1.95, 1.74	2.42, 2.32			
Lys	7.71	4.09	1.60, 1.45	1.17	1.35	3.05	7.37 NH
GABA	7.45	2.72	1.29	1.80			
Cys	7.64	4.49	3.37, 3.26				7.33, 6.83 $CONH_2$
Unit C							
Succ		2.25	2.40				
Ala-1	8.09	3.89	1.32				
Lys-2	8.15	4.28	1.69	1.20, 1.32	1.54	2.84	
Ile-3	7.17	3.69	1.77	1.15, 0.96 CH_2, 0.73 CH_3	0.61		
Tyr-4	7.43	4.35	2.98, 2.86				H-3,5: 6.68, H-2,6: 6.99
Arg-5	7.68	4.09	1.81, 1.61	1.41	3.04	7.08	
Lys-6	7.50	4.08	1.74, 1.60	1.19	1.45, 1.59	3.06, 2.81	7.52 NH
Gly-7	7.84	3.71					

[a] For convenience, the numbering of the aromatic substituent starts at the nitrogen substiutent (C-1) and is clockwise. [b] Approx. 21% *cis* proline with shifts Hα: 3.78, Hβ: 1.77, Hγ: 1.55, Hδ: 3.35, 3.22.

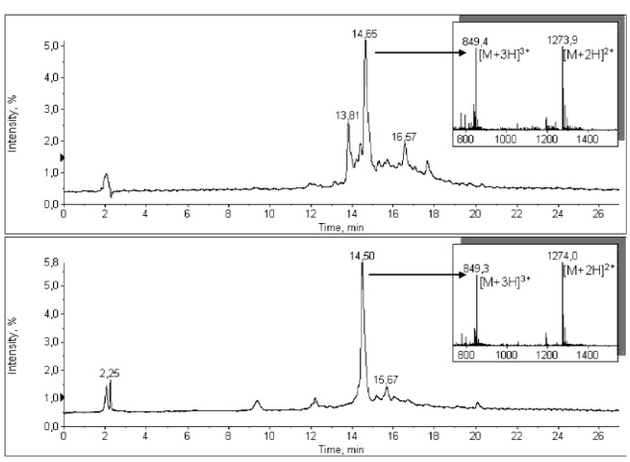


Fig. 4 HPLC chromatograms and mass spectra of crude **8** generated through fragment condensation (top; the peak with a retention time of 13.81 min represents a side product resulting from incomplete coupling of **6**), and stepwise synthesis (bottom). See experimental sections for conditions.

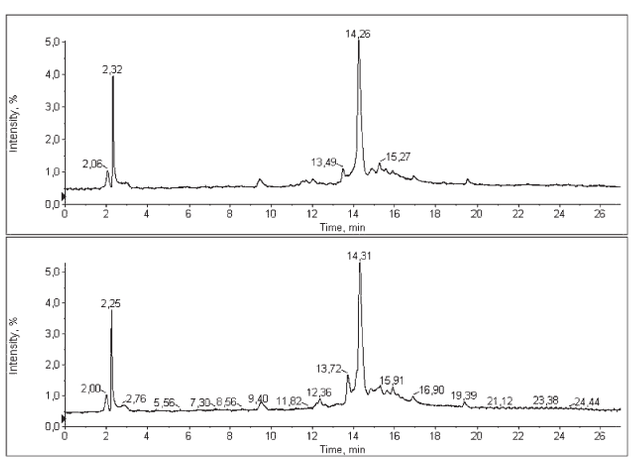


Fig. 5 HPLC chromatograms of mixed crude products from both synthesis protocols. Top: **7**, bottom: **8**. See experimental section for conditions.

NOEs. Furthermore, NOEs were observed between the succinyl methlene groups of units A and C with the neighbouring amide protons of the chain-ends of Lys, and amide protons of Tyr and Ala, respectively. These interactions clearly demonstrate the cyclic nature of units A and C. Similar interactions between the moieties of unit B established their sequence, and the cyclic structure from interactions of the β methylene protons of the cysteine residue with the aromatic system. Finally, the linkage of unit A with B was evident from the NOE between the terminal Gly NH and the aromatic system, and that between unit B and C from the (*i*, *i* + 1) NHNH NOEs of Gly of unit C with the εNH of Lys from unit B. The chemical shifts of **7** and **8** were essentially identical, except for those of the central unit B and the neighbouring glycine residues of unit A (Table 2).

Although the ESI-mass spectra of purified **7** and **8** showed only a single molecular ion peak, the NMR signals indicate two species present in solution. This is most easily seen for the lowest field signal of **8** at *ca.* 9.2 ppm, where two signals are observed corresponding to *ca.* 24% for the minor component. The nature of this second component is clear from inspection of the TOCSY spectrum in the region 4.3 to 1.5 ppm, where two sets of characteristic cross peaks for *trans* and *cis* proline moieties are evident, which upon integration indicate a content of *cis*-isomer of approximately 21%. While such a high percentage of *cis*-isomer is unusual, it has been documented previously for linear peptides containing the YPX sequence.[17]

Table 2 ^{1}H chemical shifts of **7** at 300 K in 50% aqueous CD_3CN

	HN	Hα	Hβ	Hγ	Hδ	Hε	Others
Unit A	All other residues have identical shifts to those of **8**.						
Gly-9	8.00	3.89, 3.79					
Gly-10	7.89	3.89					
Unit B							
Arom[a]	H-2: 7.50, H-5: 7.45, H-6: 7.45, NH: 9.46						
GABA	8.00	3.27, 3.17	1.82, 1.73	2.31, 2.24			
Lys	7.90	4.01	1.63, 1.54	1.25, 1.21	1.38	3.06	7.39 NH
GABA	7.68	3.10	1.61	2.10			
Cys	7.93	4.25	3.34, 3.14				7.29, 6.71 $CONH_2$
Unit C	All residues have identical shifts to those of **8**.						

[a] For convenience, the numbering of the aromatic substituent starts at the nitrogen substiutent (C-1) and is clockwise.

Conclusion

Complex scaffolded peptides presenting two different cyclic peptide fragments have been generated in high purity following either a stepwise synthesis, or a convergent fragment condensation protocol, thus providing a choice of methods for different applications. In terms of synthesis economics (time and usage of chemicals), the two strategies can be considered essentially equivalent. While stepwise synthesis does not require the separate synthesis, cleavage and work-up of pre-made fragments, and may therefore be more convenient for the synthesis of individual scaffolded peptides, convergent strategies offer the important benefit of "mixing and matching" scaffolds and fragments, enabling the generation of highly complex scaffolded peptide libraries from relatively small sets of scaffolds and fragments. The established methods are currently being used to generate scaffolded peptides presenting cyclic protein fragments, which are part of discontinuous protein binding sites, with the aim of generating inhibitors of protein–ligand interactions based on the mimicry of protein binding sites.

Experimental

Peptide synthesis

All peptides were synthesized using an automated multiple peptide synthesizer (Syro from MultiSynTech, Witten, Germany). Scaffolds **1** and **2** were generated as previously described.[7] Briefly, the linear sequences were assembled on polyoxyethylene-grafted polystyrene resin, to which the Rink amide linker was attached (TentaGel S RAM resin, 100 mg, 0.25 mmol g^{-1}). The N-terminal amino group was acylated with 2-fluoro-5-nitrobenzoic acid (**1**) and 4-fluoro-3-nitrobenzoic acid (**2**), respectively, followed by cyclization through intramolecular S_NAr, yielding a cyclic thioether. The aromatic nitro group was reduced to an amino group using tin(II) chloride, and the resulting amino group acylated with the symmetrical anhydride of Fmoc-glycine.

Protected cyclic peptides **3** and **6** were synthesized on polystyrene-2-chlorotrityl-glycine resin (50 mg, 1.11 mmol g^{-1}) using 3 eq. Fmoc-aa/DIC/HOBt for amino acid couplings. Fmoc-Lys(Alloc) was coupled as the first amino acid. The N-terminal amino group of the peptide was succinylated (4.5 eq. succinic anhydride/9 eq. DIEA in DMF, 2 × 1 h). After removal of the Alloc group ($Pd(PPh_3)_4$, 8.6 mg mL^{-1} + 1,3-dimethylbarbituric acid, 13 mg mL^{-1} in DMF/THF 5 : 2 containing 3% DIEA, 3 h under argon), peptides were cyclized on the resin through amide formation (5 eq. PyBOP/HOBt, 10 eq. DIEA, overnight), and cleaved as C-terminal acids using 20% trifluoroethanol in DCM (2 × 1 h). Peptide solutions were evaporated, and the residues dried overnight under vacuum.

For the synthesis of **7** and **8** through fragment condensation, the free amino groups of **1** and **2** were acylated with 3 eq. of **3** + 5 eq. PyBOP/HOBt/DIEA, 2 + 3 d, affording **4** and **5**, respectively. After removal of the Alloc group from the lysine side chain of the scaffolds, **6** was coupled to the free ε-amino group (5 d), affording scaffolded peptides **7** and **8**, respectively.

For the stepwise synthesis of **7** and **8**, cyclic peptide **3** was assembled on the free amino groups of **1** and **2** through consecutive couplings of the respective amino acids (5 eq. Fmoc-aa/DIC/HOBt, 2 × 1 h + capping with acetic anhydride/pyridine/DMF 1 : 2 : 3), succinylation of the N-terminus (as described above), followed by Dde-removal (2% hydrazine, 10% allyl alcohol in DMF, 4 × 7 min.), and cyclization on the resin using PyBOP/HOBt/DIEA (as described above), affording **4** and **5**, respectively. After Alloc-removal (as described above) cyclic peptide **6** was assembled at the lysine ε-amino groups of **4** and **5**, yielding **7** and **8**, respectively.

Peptides were cleaved from the resin as C-terminal amides using a mixture of TFA, DCM, water, and triisopropylsilane (70 : 20 : 5 : 5) for three hours, precipitated in a cold 1 : 1 mixture of *tert*-butylmethyl ether and cyclohexane, extracted with water, and lyophilized. Crude peptides were analyzed by RP-HPLC (column: PLRP-S 150 × 2.1 mm, particle size: 3 μm; gradient: 5–65% 0.1% TFA/acetonitrile in 0.1% TFA/water in 20 min, flow rate: 0.25 mL min^{-1}, detection: 214 nm) with online ESI-mass spectrometry detection (LC-MS). Peptides were purified by preparative HPLC on a 250 × 10 mm NUCLEOSIL RP18 column. **7**: ESI-MS ($C_{120}H_{170}N_{30}O_{30}S$) calc. 1272.625 $[M + 2H]^{2+}$, 848.750 $[M + 3H]^{3+}$; found 1272.633 $[M + 2H]^{2+}$, 848.751 $[M + 3H]^{3+}$; MALDI-MS ($C_{120}H_{170}N_{30}O_{30}S$) calc. 2544,250 $[M + H]^+$; found 2544.287 $[M + H]^+$. **8**: ESI-MS ($C_{120}H_{170}N_{30}O_{30}S$) calc. 1272.625 $[M + 2H]^{2+}$, 848.750 $[M + 3H]^{3+}$; found 1272.634 $[M + 2H]^{2+}$, 848.752 $[M + 3H]^{3+}$; MALDI-MS ($C_{120}H_{170}N_{30}O_{30}S$) calc. 2544,250 $[M + H]^+$; found 2544.212 $[M + H]^+$.

NMR spectroscopy

1D and 2D (TOCSY and NOESY; mixing times 110 and 250 ms, respectively) ^{1}H NMR spectra of **7** and **8** were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer locked to the deuterium resonance of the solvent, 1 : 1 v/v $CD_3CN : H_2O$.

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