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Synthesis of Mono-ADP-Ribosylated Oligopeptides Using Ribosylated Amino Acid Building Blocks

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Abstract: Adenosine diphosphate ribosylation (ADP-ribosylation) is a widely occurring post-translational modification of proteins at nucleophilic side chains of amino acid residues, such as asparagine, glutamic acid, and arginine. Elucidation of the biological role of ADP-ribosylation events would benefit from the availability of well-defined ADP-ribosylated peptides. Main issues in the construction of synthetic ADP-ribosylated peptides involve the availability of protected ribosylated amino acids suitable for peptide synthesis, development of a protective group strategy for peptide fragments compatible with the integrity of the adenosine diphosphate moiety, and an efficient procedure for pyrophosphate formation. In this paper we present a first approach to the chemical synthesis of ADP-ribosylated peptides in solution and on solid support. We describe an efficient synthesis of suitably protected ribosylated asparagine and glutamine building blocks suitable for Fmoc-based peptide synthesis. We further demonstrate a successful application of these ribosylated amino acids in the assembly of three fully synthetic ADP-ribosylated peptides by solution and solid phase approaches.

Introduction

Adenosine diphosphate ribosylation (ADP-ribosylation) is a post-translational modification of proteins that is effected by mono-ADP-ribosyl transferases (MARTs) or poly-ADP-ribosyl polymerases (PARPs).[1] These enzymes function by transferring ADP-ribose from β-NAD^+ to nucleophilic functional groups in the side chain of amino acid residues in the target protein, a process that is accompanied by the release of nicotinamide (see Figure 1).[2] The best studied ADP-ribosylations are related to the actions of bacteria, such as *Vibrio cholera*, *Bacillus cereus*, *Staphylococcus aureus*, *Corynebacterium diphtheria*, and *Clostridium botulinum*.[3,4] Host proteins playing a role in the immune response, cell adhesion, and metabolism are ADP-ribosylated at Asn, Glu, Asp, Arg, or Cys residues, thereby undergoing an alteration in their functioning.[1] Although ADP-ribosylating enzymes have been identified in many prokaryotic and eukaryotic species as well as in viruses, the biological processes in which they partake and their exact mode of action have not been clarified completely.[2] Interestingly it was recently proposed that inhibiting ADP-ribosylation might be a novel approach to cancer therapy.[5]

We envisage that the elucidation of the role of ADP-ribosylation events would benefit greatly from the availability of well-defined ADP-ribosylated peptides and analogues thereof. The synthesis of these compounds has not yet been described, and we thus made it the subject of our studies.

Main issues in the construction of mono- or poly-ADP-ribosylated peptides entail the synthesis of protected ribosylated amino acids that are compatible with peptide synthesis as well as an appropriate procedure for pyrophosphate formation. In this paper we present the synthesis of suitably protected ribosylated asparagine and glutamine building blocks and their application in the assembly of ADP-ribosylated peptides by solution and solid phase approaches.

Results and Discussion

The key *N*-ribosylated Asn and Gln building blocks **6** and **7** (Scheme 1) are provided with the mutually orthogonal TBDPS and Fmoc protecting groups, allowing introduction of a pyrophosphate moiety and elongation of the peptide chain in a sequential manner. The route of synthesis started with silylation of the primary alcohol of known β-D-ribofuranosyl azide **1** and subsequent acylation of the secondary 2′ and 3′ hydroxyls in **2** to give fully protected azide **3** in near quantitative yield. Reduction of β-azide **3** at 10 °C using PtO_2/H_2 resulted in the formation of an epimeric hemiaminal mixture. EDC-mediated coupling of this mixture with either *Z*-Glu-OBn or *Z*-Asp-OBn gave the ribosylated amino acids **4** and **5** as anomeric mixtures

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(6) 2,3,5-Tri-*O*-Ac-β-D-ribofuranosyl azide was prepared according to Stimac, A.; Kobe, J. *Carbohydr. Res.* **1992**, *232*, 359–365, and subsequently deacetylated using Zemplen conditions.

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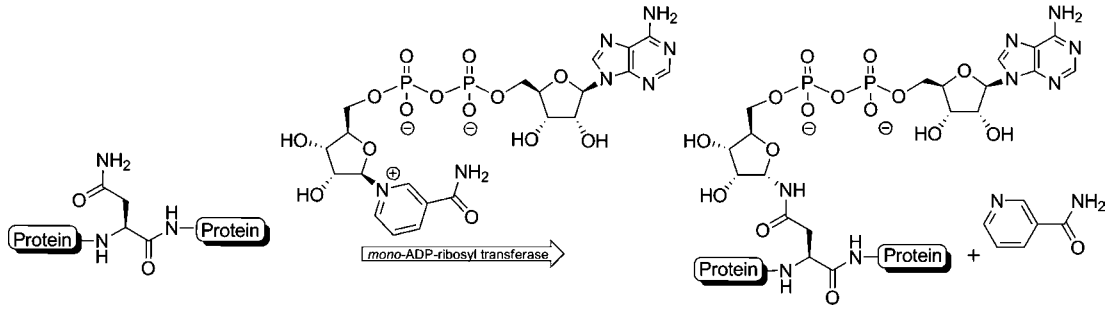
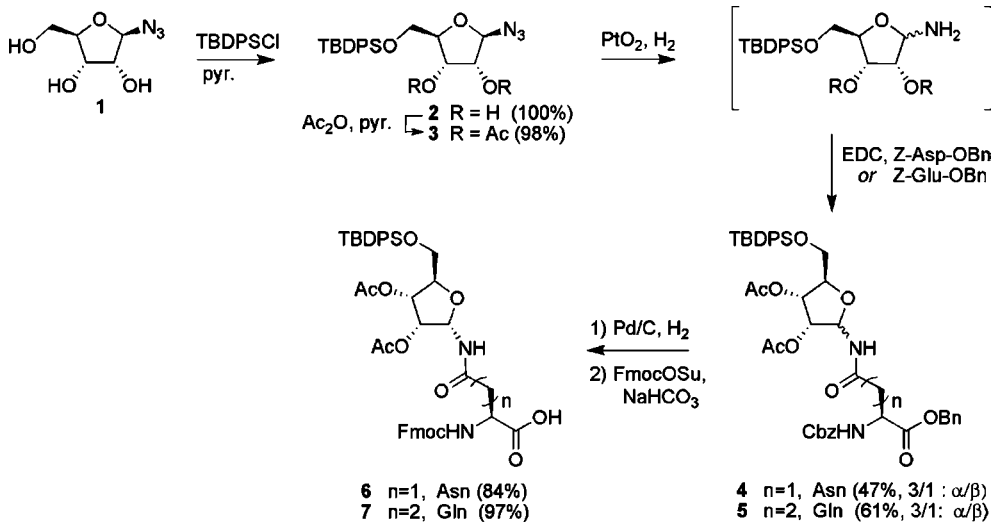


Figure 1. Example of mono-ADP-ribosylation of a protein at an asparagine residue.

Scheme 1. Ribosylated Asn and Gln Fmoc Amino Acids



(α:β = 3:1), respectively.[7] In addition a minor side reaction was detected in both cases, originating from migration of the acetyl group from the 2′ *O* to the 1′ *NH*.

After separation by silica gel column chromatography, the identities of the individual anomers of both asparagine (**4**) and glutamine building blocks (**5**) were established by means of NOESY-NMR spectroscopy.[8]

With suitably protected ribosylated Gln and Asn building blocks in hand, the synthesis of mono-ADP-ribosylated dipeptide **18** in solution was undertaken (see Scheme 2).[9] Peptide fragment **18** is derived from the mammalian Rho protein, which is ADP-ribosylated by the *Clostridium botulinum* C3 toxin at the Asn-41 residue.[10] The partially protected precursor **17** of target dipeptide **18** was provided with base labile protecting groups (Fm, Fmoc, Ac, and Cbz carbonate) to permit a straightforward alkaline deprotection procedure in the final stage of the synthesis. The route of synthesis started with the preparation of suitably protected tyrosine derivative **10** (Scheme 2) by conversion of commercially available Boc-Tyr(OBn)-OH into Fm ester **8**, followed by hydrogenolysis of the benzyl ether and finally installation of the Cbz group at the released phenolic hydroxyl function. Acidic removal of the Boc group in **10** and EDC-assisted coupling to ribosylated Asn building block **6** resulted in clean formation of dipeptide **11**. After unmasking the 5′-OH with HF/pyridine both the phosphate and the pyrophosphate functionality were introduced by adaptation of our previously published procedure.[11] Phosphate monoester **14** was obtained by phosphitylation of ribosylated dipeptide **12** with bis(*p*-methoxybenzyl)-*N*,*N*-diisopropylphosphoramidite **13** and activator dicyanoimidazole (DCI), followed by oxidation of the intermediate phosphite triester with *t*BuO_2H in nonane and finally *p*-methoxybenzyl removal using 3% TFA. Similar phosphitylation of adenosine **15** and changing the oxidation conditions to iodine in pyridine afforded the putative intermediate **16**, as an activated phosphorimidazolidate suitable for pyrophosphate formation. Addition of crude **14** to in situ formed **16** resulted in the partially protected mono-ADP-ribosylated dipeptide **17**, which was purified using RP-HPLC. Removal of Fm and Fmoc groups using DBU, followed by cleavage of the remaining acyl functions using aqueous ammonia and purification by gel filtration, provided us with 4.1 mg (4.9 μmol) of the homogeneous target compound **18**.

The successful synthesis of the mono-ADP-ribosylated dipeptide **18** prompted us to investigate an approach toward elongated peptide fragments. Having established an efficient solution phase method for the incorporation of ribosylated amino acid **6** as well as a suitable solution phase method to prepare phosphate and pyrophosphate moieties with the aid of phosphitylating agent **13**, we were curious to find out whether these methods were also applicable on solid phase synthesis. Two complementary procedures are conceivable to introduce the pyrophosphate

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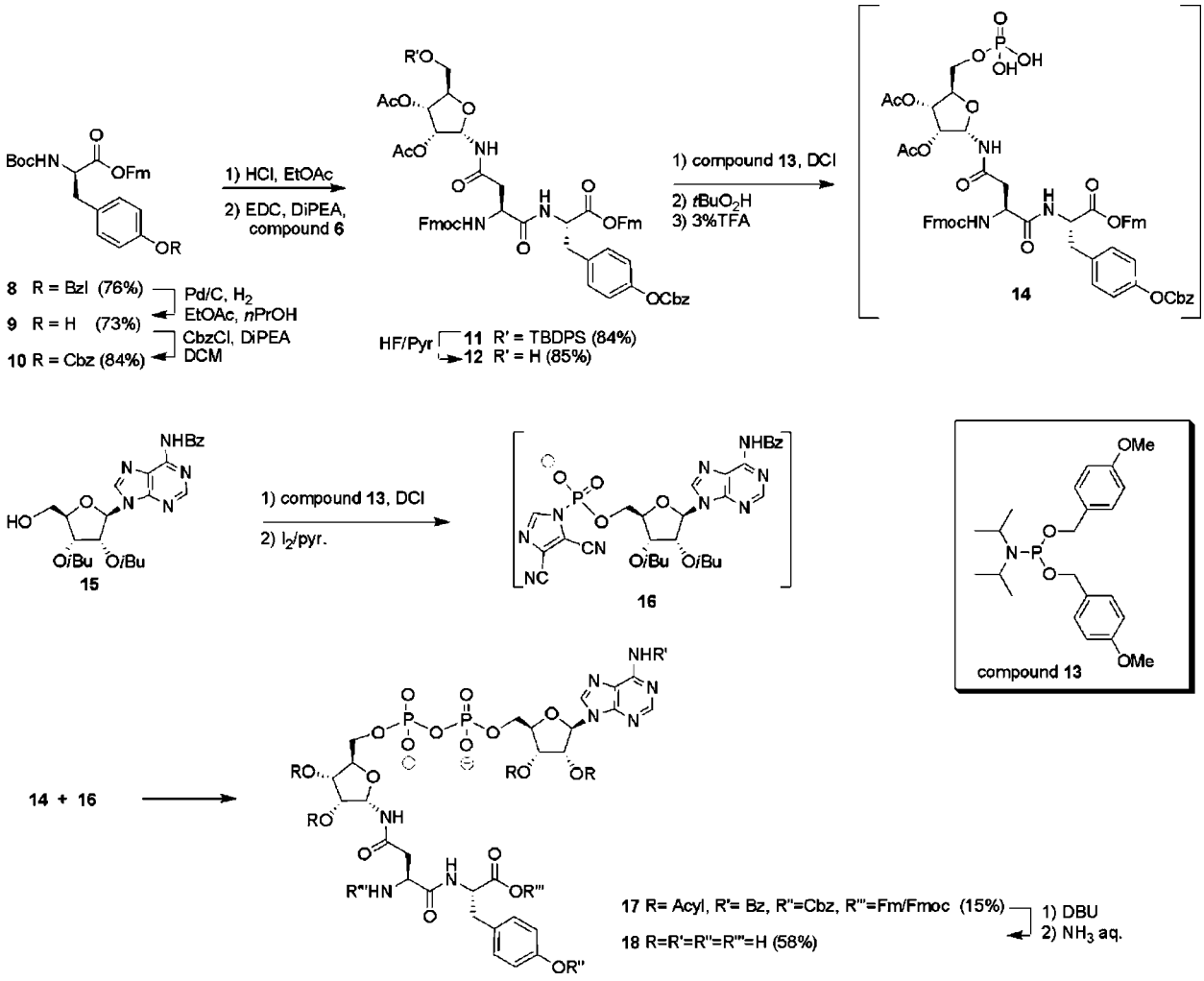
Scheme 2. Synthesis of Mono-ADP-ribosylated Dipeptide **18**



function by reacting a phosphate monoester and an activated phosphorimidazolidate. The phosphate monoester-equipped peptide can be reacted with the nucleotide phosphorimidazolidate, or by reversing functionalities, the phosphorimidazolidate of the ribosylated peptide can be reacted with the phosphate monoester of the nucleotide. ADP-ribosylated peptides **21** and **25** (see Scheme 3) were constructed each using a different approach to on-resin pyrophosphate formation.

We opted to investigate the first procedure for on-resin pyrophosphate introduction, having the reactive phosphorimidazolidate at the 5′ hydroxyl of immobilized ribosylated peptide **19** and adding the phosphate monoester in solution, on model hexapeptide **21** (see Scheme 3) containing an ADP-ribosylated Asn residue. We selected 4-hydroxymethylbenzoic acid (HMBA) as a suitable linker allowing the removal of the base labile protective groups and the release of the ADP-ribosylated peptide from the resin in a single step.[12] Hexapeptide **19** was assembled on commercially available Tentagel resin equipped with the HMBA linker using a BOP/HOBt Fmoc-based SPPS protocol.[9] En route to immobilized **19** the incorporation of ribosylated Asn building block **6** as well as ensuing repetition of the amino acid coupling cycle proceeded uneventfully, as was determined by LC-MS analysis of crude ribosylated peptide, obtained after cleavage from the solid support and removal of the protecting groups in **19** on an analytical sample.[13] Unmasking of the 5′ hydroxyl of ribose **19** with TEA/HF in pyridine permits the introduction of the ADP moiety. The activated phosphorimidazolidate was installed at the 5′ hydroxyl of the immobilized peptide **20** using DCI-assisted phosphitylation with reagent **13** and subsequent oxidation using iodine in pyridine. Addition of 6-*N*-benzoyl-2′,3′-di-*O*-isobutyryladeninyl-5′-monophosphate[11] led to formation of the immobilized and protected ADP-ribosylated peptide. Next the Dmab group on the glutamic acid residue side chain was removed by a two-step procedure,[14] and subsequent removal of the remaining protecting groups and concomitant cleavage from the resin were effected by treatment with methanolic ammonia to give C-terminal methyl ester **21**. LC-MS analysis of the crude reaction mixture showed besides the main product **21** the presence of a few side products, of which the C-terminal carboxamide, the terminal monophosphate, and the corresponding H-phosphonate could be identified.[15]

We explain the formation of H-phosphonate by invoking a partial degradation of the phosphite triester on the resin prior

(12) Furthermore HMBA is reported to be a good choice to afford thioesters, which in the future will be convenient in the synthesis of larger *m*-ADP-ribosylated peptides or proteins using native chemical ligation.

(13) See Supporting information for LC-MS traces of crude ribosylated peptides.

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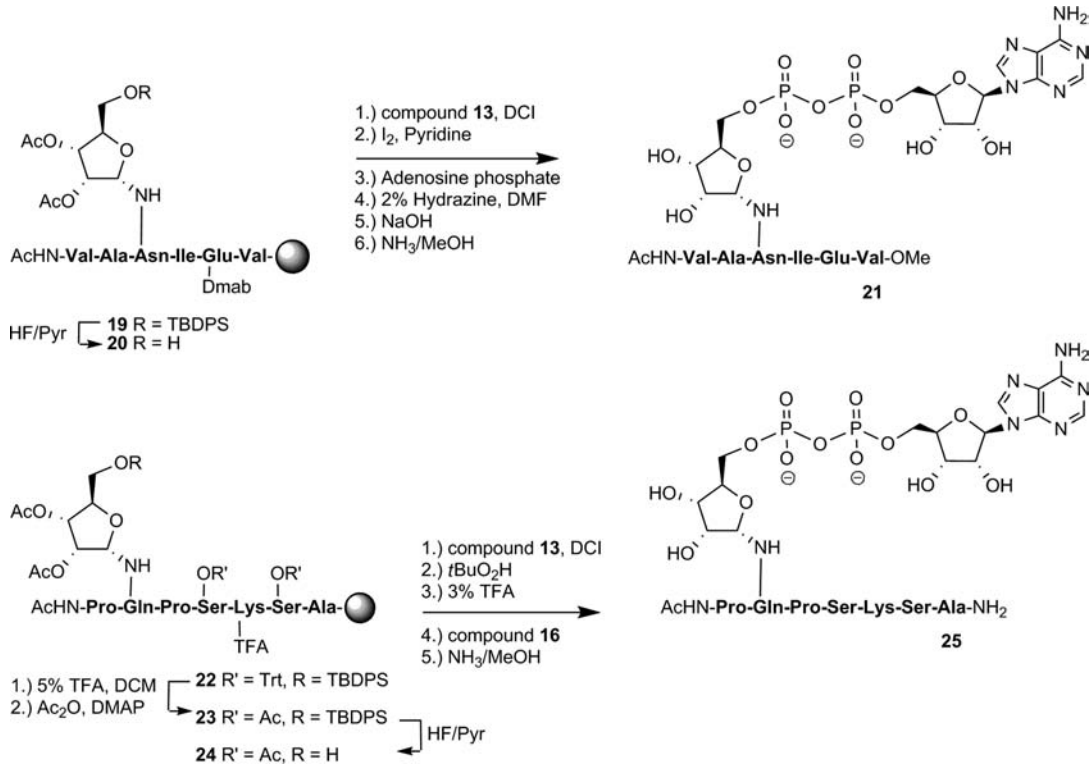
Scheme 3. Mono-ADP-ribosylated Peptides **21** and **25**



to iodine oxidation; the presence of the monophosphate was attributed to hydrolysis of the phosphorimidazolidate due to incomplete coupling of the incoming phosphate monoester. Nevertheless, 0.8 mg (0.6 μmol) of the pure mono-ADP-ribosylated hexapeptide **21** could be obtained after two RP-HPLC cycles, and the identity of the product was ascertained by ^{31}P NMR spectroscopy and high-resolution mass spectrometry.

In the second procedure to introduce the pyrophosphate linkage the phosphate monoester was installed at the 5′ hydroxyl residue of immobilized ribosylated peptide **24**, while phosphorimidazolidate **16**, prepared in solution, is added to the solid support. This has the potential to be more effective, as unwanted hydrolysis of the intermediate phosphorimidazolidate can be counterbalanced by the addition of an excess of **16**. We opted to use hexapeptide **24** (see Scheme 3), containing an ADP-ribosylated Gln residue. ADP-ribosylated heptapeptide **25** is an isosteric fragment of the naturally occurring mono-ADP-ribosylated N-terminus of human histone H2B (Scheme 3).[16] The peptide moiety is N-linked to ADP-ribose, whereas in nature the histone H2B is O-linked via glutamic acid. Precursor heptapeptide **22** was prepared according to the same procedure as described for the synthesis of **19**. Also in this case the incorporation of ribosylated Gln building block **7** proceeded uneventfully.[13] Ensuing protecting group manipulations replaced the trityl protecting group on the serine residues for the base labile acetyl and released the 5′ hydroxyl of ribose to give suitably protected peptide **24**. On-resin phosphate monoester formation was attained by phosphitylation with **13** as described above, oxidation of the intermediate phosphite triester with *t*BuO$_2$H, and *p*-methoxybenzyl removal using 3% TFA. Ensuing treatment of the resin with an excess of phosphorimidazolidate **16** gave the immobilized protected precursor of target **25**. Complete deprotection and cleavage from the solid support was effected by treatment with methanolic ammonia. Analysis of the crude mixture by LC-MS showed besides the main product **25** again the terminal monophosphate and the corresponding H-phosphonate as side products. Purification of the mixture by RP-HPLC followed by gel filtration afforded 1.3 mg (1.0 μmol) of homogeneous ADP-ribosylated heptapeptide **25**. The formation of the peptide H-phosphonate in the course of the phosphitylation reaction proved to be a significant hindrance en route to the target ADP-ribosylated peptides, and in order to suppress this unwanted transformation, we reinvestigated the phosphitylation reaction of **13** on the immobilized ribosylated peptide. Unfortunately it proved to be impossible to suppress the formation of the H-phosphonate by variation of the excess of **13** and the reaction time. Our attempts to use an alternative phosphorylation method involving the treatment of the immobilized peptide with POCl$_3$ in the presence of pyridine did not result in an efficient formation of the targeted phosphate monoester on resin.[17]

A number of biologically relevant applications can be envisaged for the peptides accessible by the methodology described here. One line of research would be the attachment of ADP-ribosylated peptides, such as the ones prepared in this study, to a carrier protein as well-defined haptens to generate antibodies against the ADP-ribose modification. Such tools might prove invaluable in the identification of mono-ADP-ribosylated cellular proteins, an intriguing but little understood post-translational modification.[18] In this fashion peptide **25** may find use in the discovery of the hitherto unknown ribosylation on the Gln side chain. In an alternative line of research, ADP-

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ribosylated peptides **18**, **21**, and **25** or closely related analogues represent well-defined substrates for elongation by enzymes of the PARP family. As certain small peptides are reported to be accepted as substrates for PARP-1 (nonapeptide) and ADP-ribose protein lyase (pentapeptide, PE(ADP-ribosyl)PAK), a better insight into the mechanism of the action of PARP enzymes can be attained by using synthetic mono-ADP-ribosylated peptides as substrates in elongation assays.[19] Mono-ADP-ribosylated peptides containing ADP-ribosylated glutamine (prepared using Fmoc amino acid **7**), if indeed accepted by PARPs as substrates, allow preparation of poly-ADP-ribosylated proteins containing a stabilized ribose−protein linkage, when combined with established chemical ligation tactics to extend the polypeptide chain.[20] Such constructs are unattainable by current biochemical techniques.

Conclusion

In conclusion we have presented a convenient method for the synthesis of suitably protected ribosylated asparagine (**6**) and glutamine (**7**) building blocks amenable to straightforward incorporation in the solution and the solid phase peptide synthesis schemes. These modified amino acid building blocks allowed us to synthesize well-defined ADP-ribosylated peptides for the first time and in milligram amounts, which can be useful for biological experiments. Further efforts will be made to improve the yield of the immobilized phosphate monoester and possibly to increase the efficiency in pyrophosphate formation. Apart from this we will also direct our research toward the synthesis of other ribosylated amino acids such as Arg, Cys, and Glu and more elaborated well-defined synthetic ADP-ribosylated peptides.

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Supporting Information Available: Spectroscopic data and experimental procedures are available free of charge via the Internet at http://pubs.acs.org.

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