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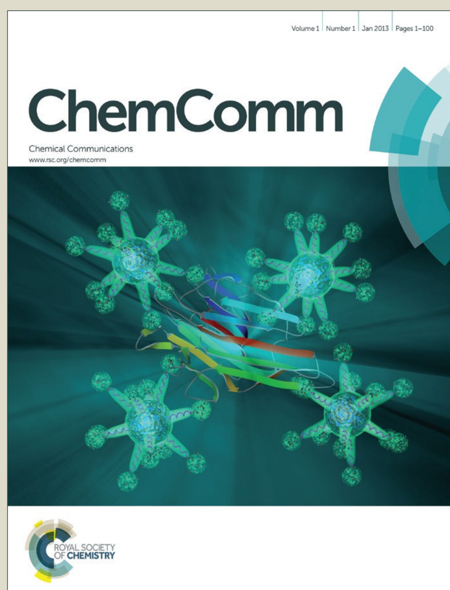

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COMMUNICATION

The Amido-Pentadienoate-Functionality of the Rakicidins is a Thiol Reactive Electrophile – Development of a General Synthetic Strategy†

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We demonstrate that a unique class-defining functionality (mc-APD) found in macrocyclic natural products with potent anti-cancer activity, imparts these compounds with electrophilic reactivity. The mc-APD group represents an interesting structural hybrid between canonical biologically relevant Michael-acceptors. Further, a novel thiol-elimination method for preparation of the mc-APD group is outlined.

Electrophilic organic compounds are a source of both fascination[1] and concern[2] within molecular pharmacology. Traditionally, small molecule electrophiles have been viewed as problematic due to their (increased potential for) off-target effects compared to unreactive compounds.[3] For some classes of electrophiles such concern is certainly legitimate,[4] however, many examples demonstrate that highly specific interactions between proteins and electrophilic ligands are also possible, thus forming the basis for a number of useful chemical probes.[1,5] Indeed several approved drugs exploit covalent mechanisms underscoring that reactive compounds can be safe.[3] Covalent modulators often show increased target residence time and high ligand efficiency.[3]

Michael acceptors constitute a major class of electrophiles and although there is significant variation within the carbon frameworks and electron withdrawing groups, privileged structures have emerged, as illustrated in Figure 1A. Microcystin-LR[6] and syringolin A[7] are classical examples of reactive natural products containing a dehydroalanine or an endocyclic α,β-unsaturated amide, respectively.

Rakicidin A (RakA, Fig. 1B) is a depsipeptide natural product with selective toxicity towards hypoxic and stem-like cancer cells.[8] A defining characteristic of this compound is the presence of an unusual vinylogous dehydroalanine functionality, 4-amido-2,4-pentadienoate (APD) which, aside from the rakicidins, only a handful of natural products has been found to possess.[9] The APD-group appears to be stable only within macrocyclic settings (mc-APD) and even under these conditions its preparative handling is non-trivial.[10]

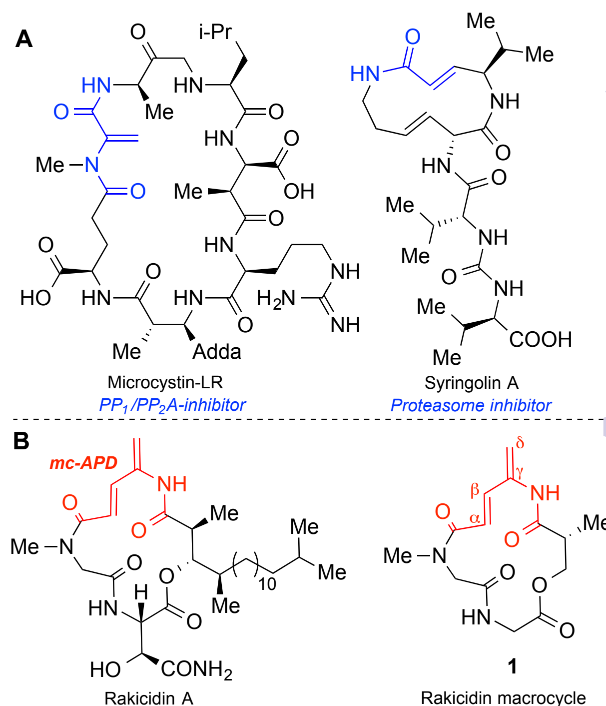

Figure 1. A. Privileged Michael-acceptor functionalities (blue) found within natural products. **B.** Chemical structures of the natural compound rakicidin A, and its synthetic surrogate **1** used in this study to test the electrophilic nature of the mc-APD functionality (red).

Although the mechanistic foundation for the biological activity of RakA is still obscure, the mc-APD has recently been demonstrated to be a critical functionality.[11] For this reason and due to its unusual chemical structure, it is of high interest to clarify the eventual reactive potential of this group. The mc-APD is expected to act as a Michael acceptor at both the β- and δ-carbons (Fig. 1B), albeit addition at the α- or γ-position (due to acyl imine tautomerism) cannot be ruled out. From the

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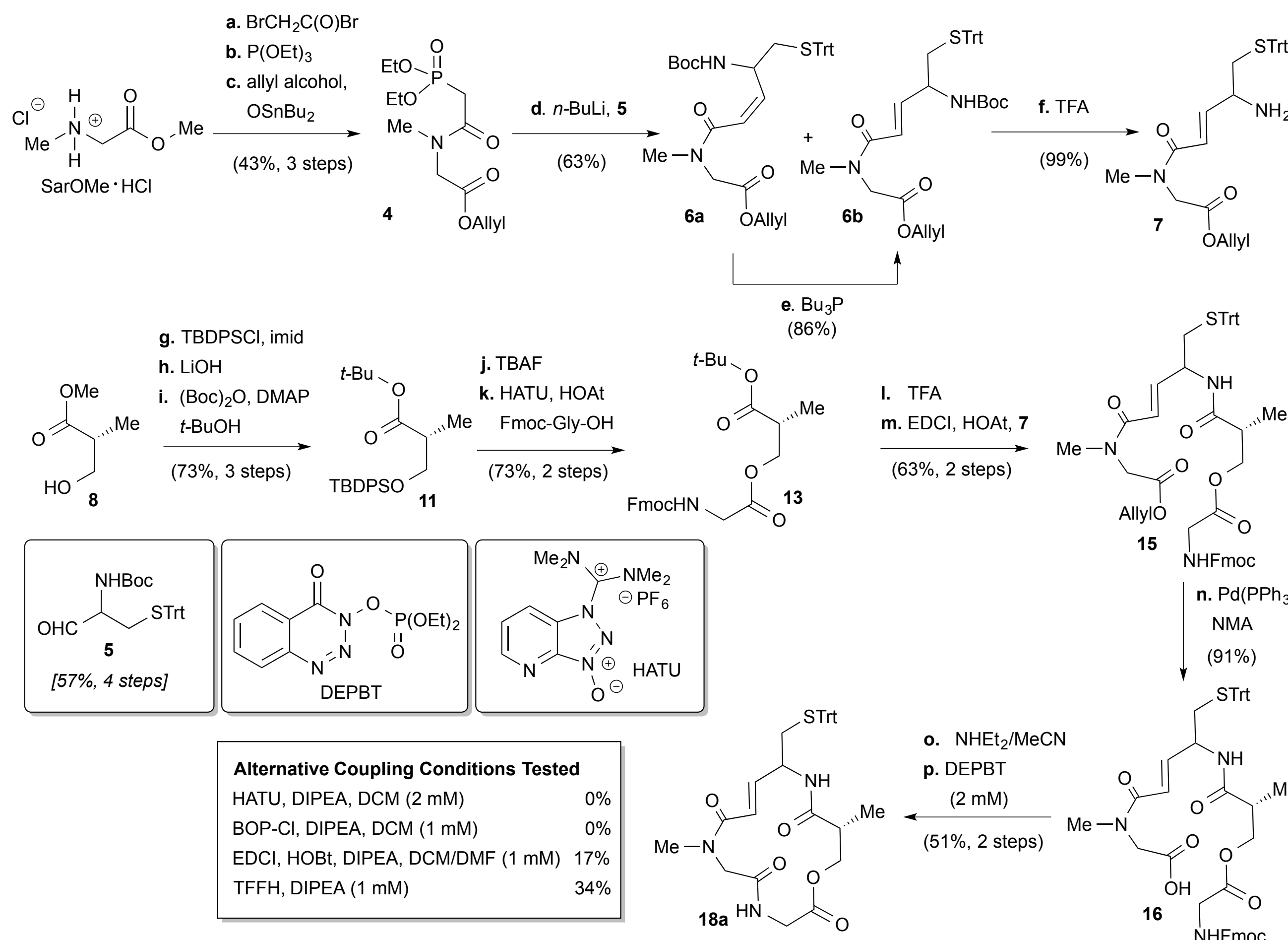
synthetic point of view, we have previously demonstrated that a serinol-derived amido-enal precursor could undergo Wittig-type transformations to prepare the APD-group in non-cyclic form.[10] This highly unstable functionality could only be handled reproducibly under dilute, neutral/slightly basic conditions but was stabilized upon subsequent macrocyclization. In their impressive recent synthesis of rakicidin A, Chen and co-workers showed that dehydration – through the intermediate mesylate – could be postponed to the last synthetic step,[12] thereby mimicking the presumed final biosynthetic operation.[13] In our hands, however, related transformations have turned out to be highly substrate-dependent and rather unpredictable.



Scheme 1. Synthesis of macrocycle **18a**. Reagents and conditions. a: $BrCH_2C(O)Br$ (1.5 equiv.), NEt_3 (3.0 equiv), CH_2Cl_2, -78 °C to -5 °C, 3 h; b: $P(OEt)_3$ (1.5 equiv.), 1,2-DCE, reflux, [illegible] h; c: Allyl alcohol, $OSnBu_2$ (1.1 equiv.), 85 °C, 24 h; d: *n*-BuLi (4.2 equiv.), THF, -78 °C, 60 min, then **5** (1.0 equiv.), -78 °C, 18 h; e: Bu_3P (1.5 equiv.), toluene, rt, 19 h; f: 15% TFA/CH_2Cl_2, rt, 45 min; g: TBDPSCl (1.35 equiv.), imidazole (3.1 equiv.), CH_2Cl_2, rt, 20 h; h: $LiOH·H_2O$ (2.5 equiv.), THF/H_2O, rt, 57 h; i: Boc_2O (2.0 equiv.), DMAP (0.3 equiv.), *t*-BuOH, rt, 90 min; j: TBAF (2.0 equiv.), THF, rt, 4 h; k: Fmoc-Gly-OH (2.5 equiv.), HATU (3.0 equiv.), HOAt (3.0 equiv.), $NEt(i\text{-}Pr)_2$ (5.0 equiv.), DMF, 0 °C, 90 min; l: 15% TFA/CH_2Cl_2, rt, 16 h; m: EDCI (1.8 equiv.), HOAt (1.8 equiv.), **13** (1.5 equiv.), **7** (1.0 equiv.), $NEt(i\text{-}Pr)_2$ (4.0 equiv.), CH_2Cl_2/DMF (4/1), 0 °C to rt, 19 h; n: $Pd(PPh_3)_4$ (0.4 equiv.), NMA (2.5 equiv.), CH_2Cl_2, 3[illegible] h; o: $NHEt_2$/MeCN (1/1), rt, 80 min; p: DEPBT (6.0 equiv.), $NEt(i\text{-}Pr)_2$ (8.0 equiv.), CH_2Cl_2/DMF (4/1), 0 °C to rt, 48 h. 1,2-DCE = 1,2-dichloroethane, THF = tetrahydrofuran, TFA = trifluoroacetic acid, DMAP = 4-dimethylaminopyridine, TBDPSCl = *tert*-butyldiphenylchlorosilane, TBAF = tetrabutylammonium fluoride, HOAt = 1-Hydroxy-7-azabenzotriazole, DMF = *N,N*-dimethylformamide, EDCI = 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, NMA = *N*-methylaniline, BOP-Cl = Bis(2-oxo-3-oxazolidinyl)phosphonic chloride, TFFH = tetramethylfluoroformamidinium hexafluorophosphate.

Thus, we hypothesized that a cysteine-residue might confer enhanced chemoselectivity as a mc-APD precursor, and therefore we developed a convergent synthesis of an appropriately protected macrocyclic precursor (Scheme 1). Boc-protected amine **6b** was constructed through a HWE-reaction between phosphonate **4** and protected racemic cysteinal[14] **5**. Following some optimization, we found that the desired HWE-reaction could be carried out at low temperature to yield a 1:1 cis/trans mixture, and that the undesired cis-isomer **6a** could be easily equilibrated in the presence of Bu_3P. Ester **13** was made in 5 steps from alcohol **8** and following removal of the *t*-Bu group, the acid was coupled to amine **7** using EDCI/HOAt in 63% yield over 2 steps. Proceeding towards macrocyclization, the allyl and Fmoc-groups were then sequentially removed and gratifyingly we found that the macrolactamization could be effected by DEPBT to afford **18a**

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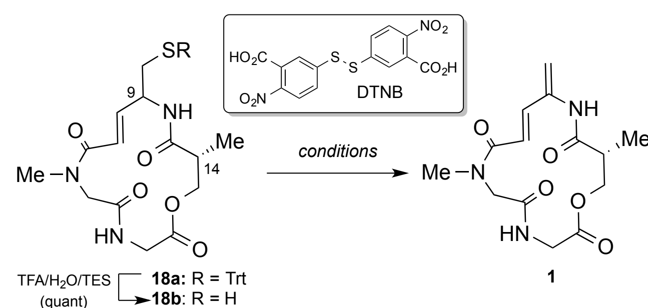
in a yield of 46% over the three steps. Several canonical coupling-systems proved ineffective or failed completely when tried in this transformation. Having achieved the desired elimination precursor, the stage was set to test the thiol-elimination conditions using Ellman's disulfide reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB), originally developed for dehydroalanine-syntheses (Table 1).[15] The trityl-group could be readily cleaved under standard conditions and the free thiol **18b** was purified by a simple precipitation. Several attempts were made with and without ancillary base to first generate the initial mixed disulfide, which by TLC analysis appeared to proceed smoothly. However, subsequent attempts at inducing reduction-elimination under neutral conditions with HMPT (Table 1, entries 1-2) were unsuccessful, and base-mediated elimination employing DBU only resulted in small quantities of the product (Table 1, entries 3-4).

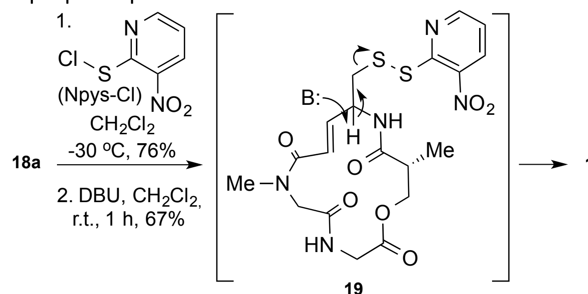
Table 1. Thiol-elimination to form the mc-APD system.



Entry	Conditions[a]	Yield (%)[b]
1	DTNB, NEt_3, DMF then HMPT	0
2	DTNB, NEt_3, CH_2Cl_2/DMF then HMPT	0
3	DTNB, DMF then DBU	11
4	DTNB, CH_2Cl_2/DMF then DBU	12
5	Npys-Cl, CH_2Cl_2 then HMPT	0[c]

[a] All reactions were carried out in a one pot operation at ambient temperature. [b] Isolated yield. [c] Only **18b** obtained. HMPT = hexamethylphosphorous triamide, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, TES = triethylsilane.

It should be noted that we intentionally prepared the macrocycle as a mixture of C9-epimers [the additional stereogenic center at C14 was arbitrarily chosen as (*R*)] in order to accommodate the possibility that the efficiency of thiol-elimination might differ for the two diastereomers. Following these surprising shortcomings with DTNB, we turned our attention to alternative reagents and indeed found that efficient disulfide formation could also be achieved using 3-Nitro-2-pyridinesulfenyl chloride (Npys-Cl, Table 1, entry 5). This reagent was initially developed as a protecting group for alcohols and amines,[16] being later on extended to thiols,[17] but to the best of our knowledge has never been employed in thiol-eliminations. The Npys-mixed disulfides are easily handled and can be purified by chromatography. Exposure of the Npys-disulfide to HMPT resulted cleanly in deprotection to form **18b** (Table 1, entry 5) indicating that the nucleophilic phosphine preferentially attacks the sulfur-atom distal to the macrocycle. Encouraged, however, by the easy formation of the Npys-disulfide, we took advantage of a further feature of this reagent: Its ability to directly exchange with the trityl protecting group.[18] Thus, exposure of compound **18a** to NPys-Cl at -30 °C overnight allowed for direct generation of the mixed disulfide **19** in good yield (Scheme 2). Surveying conditions for the final elimination, we found that exposure of the mixed Npys-disulfide to DBU at ambient temperature furnished the mc-APD-containing macrocycle **1** in good yield after prep-TLC purification.



Scheme 2. Successful thiol elimination via the unsymmetrical disulfide intermediate **19**.

Next we evaluated the potential reactivity of macrocycle **1** towards biological nucleophiles using bovine serum albumin (BSA) as a model protein. We incubated BSA with **1** under different conditions and subsequently alkylated with iodoacetamide. Following tryptic proteolysis, cysteine-residues modified with **1** were identified by mass spectrometry. Although BSA contains a single free cysteine residue, no modified peptides were detected following a 2 hour exposure of the native protein to 100 μM of **1**. We therefore denatured and reduced the protein to expose all disulfide-bound cysteines before incubation with **1** which resulted in the detection of several modified peptides (Figure 2A-B and Table S1). In this experiment all modified peptides were identified based on their mass and sequences obtained by ms/ms (Supplemental Figure S1 and Table S1)

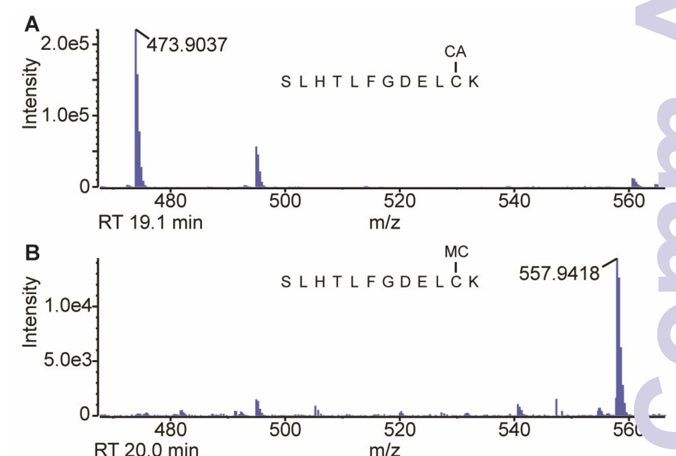


Figure 2. Electrophilic reactivity of macrocycle **1**. **A-B.** LC-MS data of the BSA-derived tryptic peptide SLHTLFGDELCK modified with either iodoacetamide (CA) or the macrocycle **1** (MC) . The peptide is identified as a triple charged species at A) 473.9 m/z (CA) and B) 557.9 m/z (MC) corresponding to a mass difference of 252 Da equivalent to the mass difference between iodoacetamide and **1**. The identity of the peptide and position of the modification were confirmed by LC-MS/MS data (Supplemental Figure S1 and Table S1).

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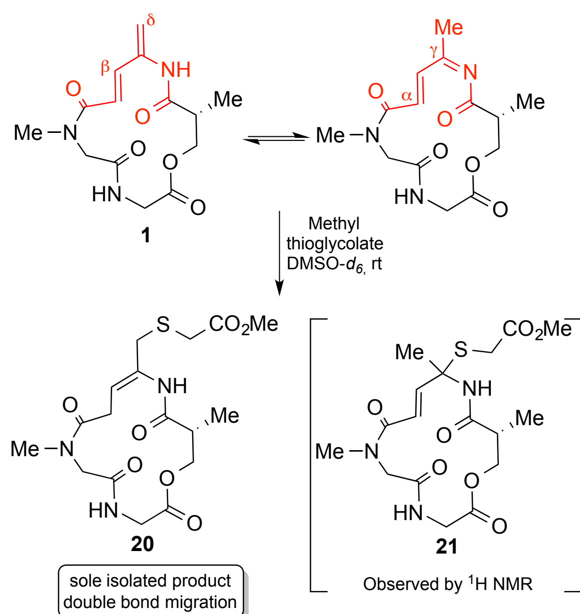
Of the 35 cysteine residues in BSA, 19 were identified carrying the macrocyclic modification (Table S1). We also studied the reactivity of **1** towards thiol nucleophiles under preparative conditions with methyl thioglycolate as a model nucleophile (Scheme 3). The reaction was carried out in DMSO-d_6 and was monitored periodically by ^{1}H NMR spectroscopy. Curiously, after 24 hours we observed predominant formation of a compound that we tentatively assign to be **21** (see supplementary information) as well as the 1,6-addition product **20** as a minor component. Compound **21** likely results from thiol-addition to the γ-carbon atom of the mc-APD, presumably through intermediacy of the acyl-imine tautomer**.** After 72 hours, however, adduct **20** was the sole product observed, perhaps indicating a reversibility of **21** back to its precursor. In accord, we were unable to purify compound **21** when this reaction was stopped at early time points, whereas compound **20** could be purified and characterized. Acyl-imine tautomerism in dehydroalanine functionalities is known, however (reversible) addition of simple thiols to such systems is to the best of our knowledge unprecedented.[19] Collectively, these experiments demonstrate the reactivity of the mc-APD group towards thiol-nucleophiles.



Scheme 3. Preparative reaction between macrocycle **1** and methyl thioglycolate.

In conclusion, we have reported a new method for the formation of the mc-APD group found in a number of depsipeptide natural products, including the rakicidins. Our discovery of the superior ability of the Npys-group to facilitate thiol elimination to construct the mc-APD system is notable, and we anticipate that this reaction will enable construction of focused mc-APD-containing compound libraries. The versatility of Npys-Cl is currently underappreciated and additional applications of this reagent are warranted. Finally, we have characterized an inherent reactivity of the mc-APD group towards thiol nucleophiles, including protein-resident cysteine residues and we speculate that electrophilic reactivity may play a key role in the interactions between mc-APD-containing natural products and their, as of yet, unknown biological targets.

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