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Synthetic Approach to Argpyrimidine as a Tool for Investigating Nonenzymatic Posttranslational Modification of Proteins

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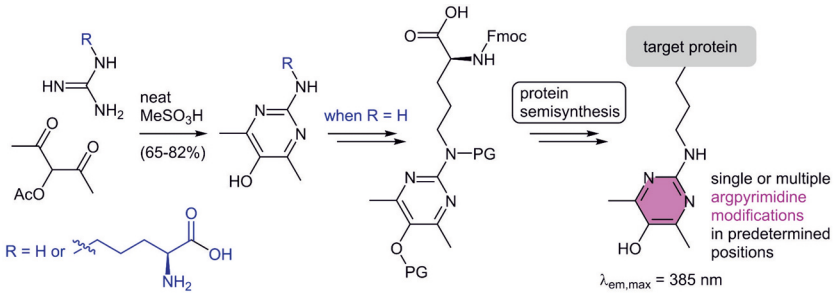

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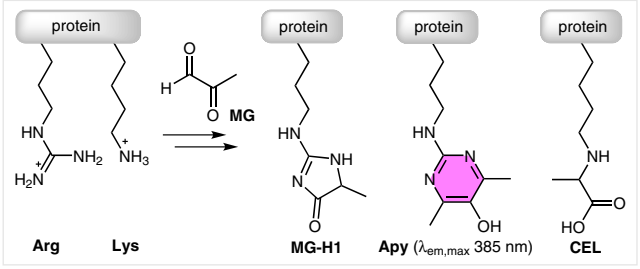
Abstract Nonenzymatic posttranslational modifications (nPTMs) of proteins are involved in age-related, metabolic and other diseases and need to be investigated at the molecular level. Here, we describe how we used organic synthesis to enable the study of the effect of argpyrimidine (Apy), an nPTM that forms at arginine residues, on one of its target proteins. We developed an efficient approach to Apy as a universal building block for Fmoc-based solid-phase peptide synthesis that allows for the construction of peptides containing this nPTM in predetermined positions. Moreover, a straightforward one-step synthesis of protecting-group-free Apy was achieved, which enabled the preparation of gram-quantities of this noncanonical amino acid that can serve as a biomarker or a feedstock in construction of Apy-containing proteins via the expanded genetic code methods.

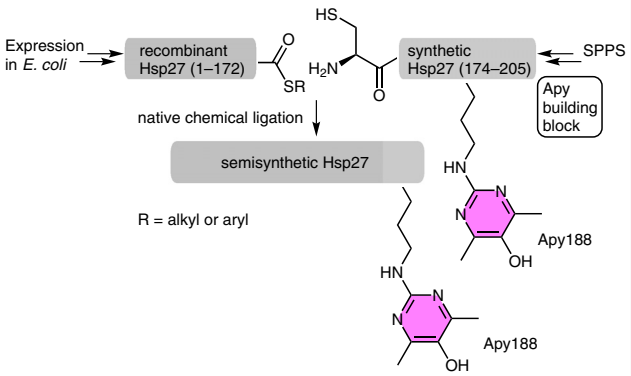
Key words protein modification, argpyrimidine, amino acid synthesis, 2-aminopyrimidinol, Mitsunobu reaction, SPPS, protein semisynthesis

Nonenzymatic posttranslational modifications (nPTMs) result from the addition of carbonyl-containing metabolites to nucleophilic side chains of proteins and are involved in age-related, metabolic, cardiovascular, neurodegenerative diseases and cancer.[1] Despite the lack of enzymatic assistance, these modifications often accumulate site-specifically,[2] and therefore homogeneous modified proteins are needed to assess the precise impact of nPTM formation. Here, we focused on argpyrimidine (Apy, Scheme 1), a fluorescent ($\lambda_{em,max}$ = 385 nm) nPTM that forms on arginine residues[3] and has been detected in aged and cataract-affected lenses,[4] in amyloid fibers from patients with familial amyloidotic polyneuropathy,[5] and in human cells and tissues from diabetic and cancer patients.[6] In order to investigate the structural and functional consequences of Apy formation on one of its in vivo target proteins, Hsp27, we devised a semisynthesis approach (Scheme 2)[7] that hinged on a suitable Apy building block for the construction of a 33-mer peptide containing this modification instead of Arg188, a position that is affected in human cancer cells.[6b] The required building block had to be suitable for standard automated Fmoc-strategy solid-phase peptide synthesis (SPPS) and as such contain a free α-carboxyl moiety, Fmoc-masked α-amine and side chains that are protected with acid-labile groups that are resistant to basic conditions used in the repeated cycles of Fmoc deprotection. By contrast, the available syntheses of Apy at that time were limited to providing protecting-group-free compounds on analytical scale.[3,8] Therefore, a novel route had to be established, capable of delivering multigram quantities of the Apy building block from readily available materials and using conventional protecting groups that do not require additional peptide manipulation.



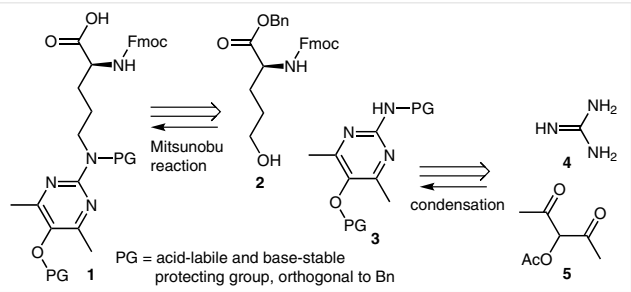
Scheme 1 Examples of nPTMs caused by methylglyoxal (MG), a by-product of glycolysis: arginine-derived MG-hydroimidazolone (MG-H1) and argpyrimidine (Apy), and lysine-derived carboxyethyllysine (CEL)[9]

We chose to construct the proposed building block **1** via a Mitsunobu reaction[10] between the known reduced glutamate derivative **2**,[11] and a suitably protected aminopyrimidinol **3** (Scheme 3). The protecting group on the amino function of the aminopyrimidine intermediate **3** had to serve an additional and critical role of acidifying the *N*–H



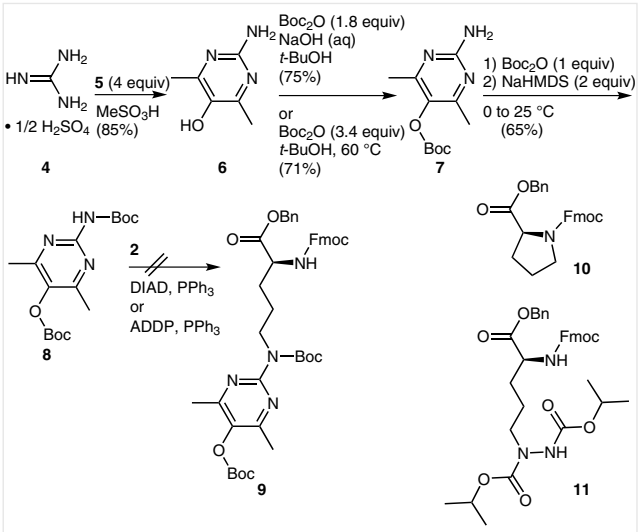
Scheme 2 Semisynthetic approach to Hsp27 with Arg188 Apy modification[7]

hydrogen sufficiently to be amenable to removal by the hydrazide anion formed in the Mitsunobu protocol. The aqueous pK_a of 2-aminopyrimidinol was reported to be ca. 20.5,[12] and thus the carbonyl function of a Boc group was anticipated to reduce it sufficiently for the reaction, particularly as the related *N*,*N*′-bis(*tert*-butyloxycarbonyl)guanidine is a known nucleophile under the Mitsunobu conditions.[13]



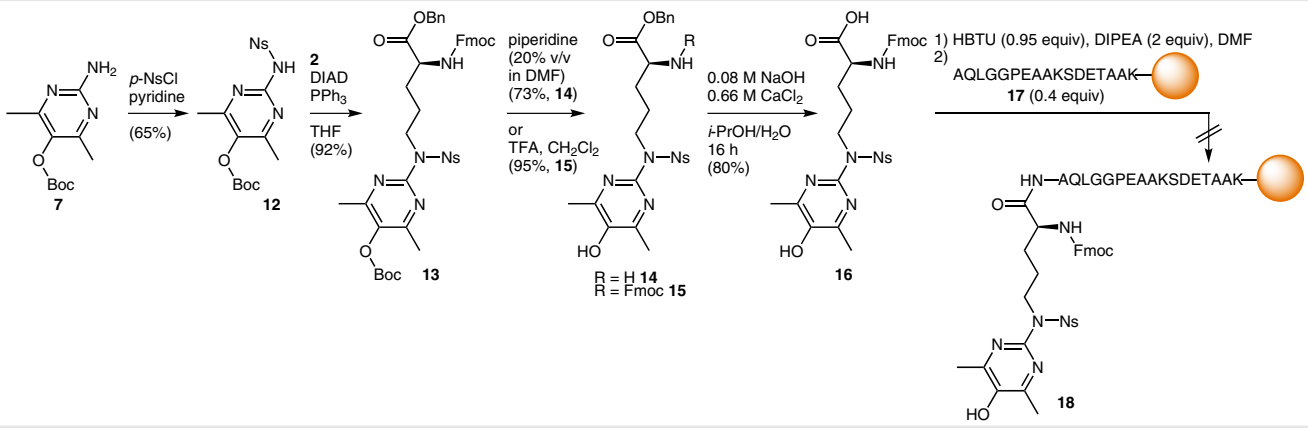
Scheme 3 Synthetic approach to the Apy building block for Fmoc-based SPPS

The synthesis began with the construction of the 2-aminopyrimidinol (**6**) by subjecting readily available guanidine sulfate (**4**) to an excess of the known 1,3-diketone **5** (available in one step from a commercial material),[14] in the presence of NaOAc according to a published method[15] (Scheme 4). In our hands, the base-mediated process did not lead to acceptable yields of the sought-after aminopyrimidinol (maximum 15% yield). Using inorganic or organic bases of varying strength (NaOAc, K_2CO_3, pyridine, NMM, Et_3N), increasing the amount of the base-labile diketone reagent to as much as six equivalents (stepwise addition), and including the use of conventional or microwave heating (up to 150 °C) in several solvents did not improve the yield of the water-soluble product **6**. The insolubility of guanidine salts (sulfate, chloride) in organic solvents, coupled with the instability of the diketone reagent to basic conditions were the major obstacles in this process. We then turned our attention to acid catalysis for the condensation reaction. Gratifyingly, using a strong but easy-to-handle methanesulfonic acid as a solvent, which completely dissolves the substrates and catalyzes the reaction, a full conversion of guanidine sulfate (**4**) into the corresponding 2-aminopyrimidinol (**6**)[8c,15,16] was achieved, and this material was isolated in 85% yield on multigram scale. While the phenol function within this last compound was smoothly converted into the corresponding *tert*-butyl carbonate (compound **7**, 75%), the next planned step of Boc protection of the aniline moiety was complicated by the persistent formation of the *N*,*N*-bis-Boc carbamate (e.g., using Boc_2O/Et_3N/DMAP) due to the acidity of *N*–H within the initially formed carbamate. Unfortunately, switching to a weaker base pyridine did not improve the desired reaction, while attempts to remove one of the two *N*-Boc groups (NaOMe/MeOH)[17] or using heat and high vacuum were also unfruitful, resulting in varying amounts of the starting material and the completely *N*-deprotected compound **7**. Fortunately, applying the modified procedure[18] of the established method involving the addition of two equivalents of NaHMDS,[19] the desired intermediate was obtained in 65% yield.



Scheme 4 Construction of the 2-aminopyrimidinol core and attempted Mitsunobu reaction

With compound **8** in hand, we were able to test the proposed Mitsunobu reaction with the primary alcohol **2**, obtained in one reduction step from the commercially available acid Fmoc-Glu-OBn.[11] Unfortunately, the intermediate **8** failed to engage in the proposed reaction, even when the more strongly basic reagent derived from 1,1′-(azodicarbonyl)dipiperidine (ADDP)[20] was used in place of the standard diisopropyl azodicarboxylate (DIAD, Scheme 4). Instead, only the cyclized material **10** and compound tentatively assigned as **11**, the product of the attack of the DIAD-derived hydrazide anion at the alkoxyphosphonium intermediate,[21] were isolated. In another attempt at the desired



Scheme 5 Successful Mitsunobu reaction and attempted peptide synthesis using the building block **16**

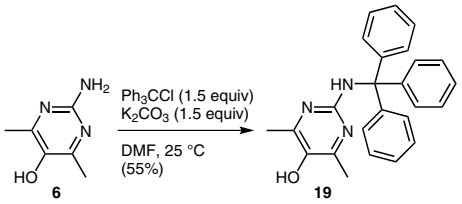
alkylation, alcohol **2** was converted into the corresponding iodide (using I_2/Ph_3P/imidazole, 97%, see Supporting Information for details), but this last compound then failed to undergo a nucleophilic attack by either amine **7** (combined with heating and weak amine bases to remove HI) or carbamate **8** (combined with strong non-nucleophilic bases to deprotonate the *N*H).

We thus turned our attention to strongly electron-withdrawing groups for the activation of the amine function toward alkylation. Indeed, *p*-nitrobenzenesulfonyl (Ns) amide **12** underwent a smooth Mitsunobu reaction to give fully protected argpyrimidine **13** (Scheme 5). To test the stability of the Ns group within this last compound, it was submitted to the standard Fmoc-cleavage conditions used in SPPS (piperidine, 20% v/v in DMF). While the Ns group was untouched, the Boc group was cleaved completely to give phenol **14**. In the hope that the methyl groups would hinder *O*-acylation of this compound while also blocking the electrophilic substitution at the 3-benzyl position, we decided to test the free-OH compound as a potential building block for Fmoc-SPPS. To this end, the *O*-Boc group was removed using TFA, and the benzyl ester within the resulting Fmoc-protected compound **15** had to be cleaved to reveal the free carboxylic acid. Attempted hydrogenolysis, mediated by palladium on charcoal, was successful in this respect but resulted in the additional reduction (mixture of partial and complete) of the aromatic nitro group (see Figure S1 in the Supporting Information). Fortunately, applying the conditions for preserving the Fmoc group during ester hydrolysis,[22] the required acid **16** was isolated in 80% yield.

This material was then submitted to the standard manual peptide coupling procedure (2.5 equiv of the amino acid **16**, activated with HBTU or HATU and DIPEA) with the preassembled peptide **17** comprising the C-terminus of Hsp27 and containing the free N-terminal amine (Scheme 5).

Unfortunately, the LC–MS chromatogram of the crude peptide obtained following the coupling reaction (Figure S2 in the Supporting Information) indicated that the major product was the unreacted peptide substrate, and only traces of the desired coupled product were present, suggesting that the free OH group was potentially interfering with the coupling step.

In an attempt to block the phenol moiety, the two most common Fmoc-based SPPS side-chain protecting groups were investigated, *tert*-butyl (*t*-Bu) and trityl (Trt). Unfortunately, the phenol function of both the fully assembled compound **15** and the aminopyrimidinol **6** were resistant to several alkylating conditions (*t*-BuBr or TrtCl/amines; TrtCl/$ZnCl_2$/Et_3N;[23] TrtOH/acids) or the decarboxylative etherification with Boc_2O/$Mg(ClO_4)_2$.[24] On the other hand, using a combination of Cs_2CO_3 or K_2CO_3 and TrtCl in anhydrous organic solvents, up to 55% of the monotritylated product could be isolated following the complete consumption of the starting material **6**. Unfortunately, this material was identified as the *N*-Trt compound **19** (Scheme 6), indicating that in this case the steric hindrance of the neighboring methyl groups overpowered the nucleophilicity distribution observed earlier for the Boc protection. The structure of compound **19** was established unambiguously through a single-crystal X-ray analysis (Figure 1).



Scheme 6 Attempted phenol protection using the trityl group

The ultimate approach (Scheme 7) involved the protection of the phenol as a TBS ether,[26] and substitution of the nonstandard Ns group with the acid-labile 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf), which is rou-

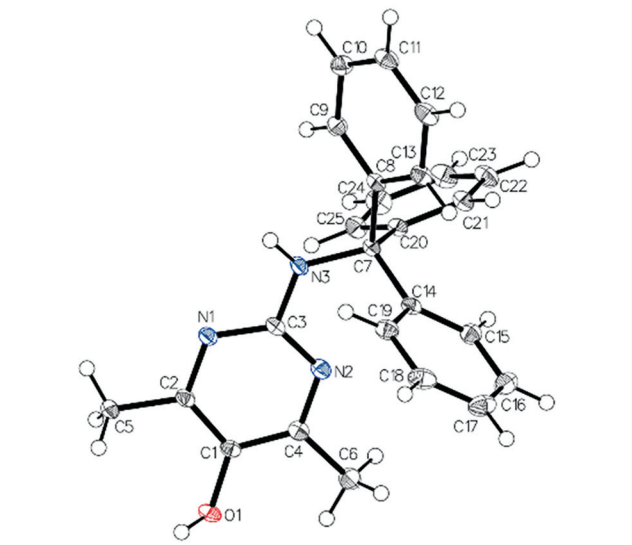
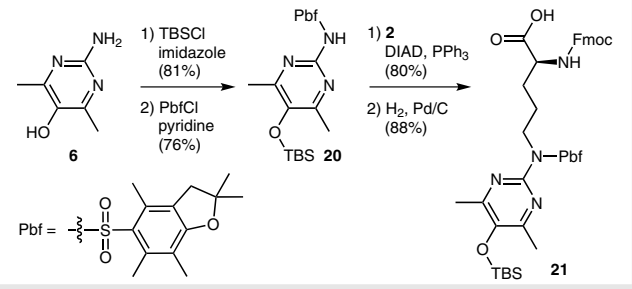


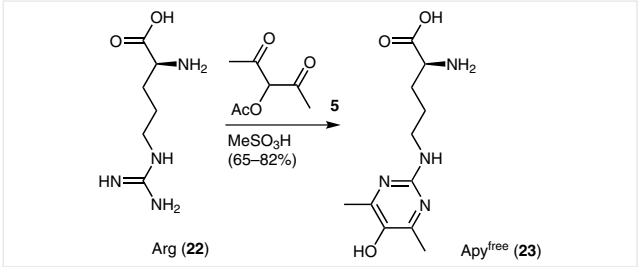
Figure 1 ORTEP diagram resulting from the X-ray crystal analysis of **19**[25]

tinely applied in Fmoc strategy SPPS. The resulting sulfonamide **20** underwent an efficient Mitsunobu reaction (80%, ca. 4 mmol scale), and the ensuing compound was submitted to gentle hydrogenolysis of the benzyl ester, carefully avoiding the side reaction of Fmoc group removal, to give the Apy building block **21** (> 5 g, five steps from guanidine).[7] This material was successfully utilized, without any deviations from the standard automated Fmoc-based SPPS protocols, in the preparation of sufficient quantities of the required peptide that enabled the semisynthesis of Hsp27 bearing the Apy188 modification.[7]



Scheme 7 Optimized synthesis of the building block **21**[7]

Samples of the free amino acid Apy are also valuable and have been previously obtained on small scale for analytical purposes via the global deprotection of **21**.[7] A more efficient and straightforward synthetic procedure for this amino acid had to be developed that could provide multigram quantities of this material. The pioneering published approaches involved the condensation of diketone **5** or its analogues with α*N*-protected or free L-arginine but required the use of corrosive materials such as 12 M HCl or purification by HPLC, limiting them to small amounts of the amino acid.[3,8a,b] After screening several mild reagents (e.g., volatile mild acids such as AcOH or hexafluoroisopropanol) and the use of heat to promote this condensation, the optimum procedure was established that involves the reaction of **5** with unprotected L-arginine **22** in methanesulfonic acid (Scheme 8).[27] In this way, argpyrimidine is obtained in up to 82% yield and on gram scale for the first time. The spectral and physical data derived from this amino acid are in excellent agreement with the literature values.[3,8] In particular, the characteristic excitation and emission maxima at 320 and 385 nm, respectively,[3] are observed in the fluorescence spectrum of synthetic Apy^{free} (**23**, see Figure S3 in the Supporting Information). With the efficient one-step synthesis of **23** in hand, it would be interesting to explore the conversion of this compound into a building block of type **1**. The main concern would be the installation of the protecting groups given the presence of several reactive moieties within compound **23**.



Scheme 8 Optimized synthesis of argpyrimidine as a free amino acid

In summary, we have developed an efficient strategy to assemble a universal Apy building block applicable to manual or automated Fmoc-based SPPS, which allows for the synthesis of peptides and proteins containing this nPTM in predetermined positions. Such compounds enable the investigation of the impact of nPTMs on the molecular properties of the affected proteins. In addition, the spectroscopic features of Apy and its relatively small size may be taken advantage of by the incorporation as a fluorescent protein label, with minimal perturbation of the structure and function of the protein to be investigated. Finally, the straightforward one-step procedure for the synthesis of Apy amino acid has enabled access to multigram quantities of this valuable material, which can be used as a standard for monitoring soluble nPTM concentrations in biological fluids or for ribosomal synthesis of Apy-derivatized proteins following reprograming of expression systems.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0036-1588225.

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(25) CCDC 1528587 for 19 contains the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.

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(27) **(*S*)-2-Amino-5-[(5-hydroxy-4,6-dimethylpyrimidin-2-yl)amino]pentanoic Acid** (**Apy^{free}**, **23**)

An adaptation of the original published procedure was employed.[8a,b] A magnetically stirred solution of L-arginine (**22**, 3.0 g, 17.2 mmol) in methanesulfonic acid (12 mL, 1.5 M) maintained at 25 °C was treated with crude diketone **5** (3.5 g, ca. 22 mmol), resulting in a mildly exothermic reaction. Further portions of compound **5** (2 × 2.7 g, ca. 17 mmol each) were added after 3 and 6 h, respectively. The ensuing dark brown viscous mixture was stirred for 48 h then cooled to 0 °C and neutralized by the dropwise addition of NH_4OH (ca. 20 mL of a 28–30% aq solution). The resulting brown-orange mixture (pH ~7) was stirred at 25 °C for 30 min then diluted with H_2O (25 mL) and loaded, using additional H_2O, onto a column of C_{18}-reversed-phase silica gel (10 × 10 cm) that had been equilibrated with MeOH then H_2O. Elution with 0 → 10 → 20% v/v MeOH–H_2O and concentration of the relevant fractions containing fluorescent material (R_f = 0.2 in 1:2:7 v/v/v H_2O–*i*-PrOH–EtOAc) afforded the title compound **23** (2.83 g, 65%) as a white powder. A portion of this material was lyophilized from TFA (0.1% in H_2O) to obtain compound **23** (zwitterion, white fluffy powder) that was used for characterization and all spectroscopic measurements; mp 192–196 °C (decomp.) [lit. for HCl salt[8a] 207 °C

(decomp.)]. $[\alpha]_D$ +28.1 (*c* 0.3, H_2O) [lit.[8a] +17.5 (*c* 0.5, 1 M HCl)]. ^{1}H NMR (600 MHz, D_2O): δ = 3.73 (t, *J* = 6.1 Hz, 1 H), 3.44 (t, *J* = 6.8 Hz, 2 H), 2.39 (s, 6 H), 1.93–1.84 (m, 2 H), 1.75–1.60 (m, 2 H). ^{1}H NMR (600 MHz, $(CD_3)_2SO$): δ = 8.14 (br s, 2 H, NH_2), 7.87 (br s, 1 H, OH), 6.38 (s, 1 H, NH), 3.86 (t, *J* = 6.2 Hz, 1 H), 3.18 (dd, *J* = 12.0, 6.2 Hz, 2 H), 2.18 (s, 6 H), 1.84–1.71 (m, 2 H), 1.63–1.49 (m, 2 H). ^{13}C NMR (150 MHz, D_2O): δ = 174.4, 150.8, 137.7, 54.3, 40.4, 27.5, 23.9, 16.8 (due to H/D exchange of the phenolic OH, the corresponding *ipso* carbon is not visible in the spectrum). ^{13}C NMR (150 MHz, $(CD_3)_2SO$): δ = 171.2, 156.3, 155.1, 138.8, 52.1, 40.3, 27.8, 24.9, 19.0. ESI-HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{11}H_{18}N_4O_3$: 255.1452; found: 255.1448.

On 0.6 mmol scale and using pre-packed C_{18} cartridges for purification, compound **23** was obtained in 82% yield.