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A chemoselective ligation for the synthesis of amino acid derivatives of virginiamycin M_1

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Abstract—An efficient chemoselective ligation approach using an oxime bond was developed for the synthesis of amino acid derivatives of virginiamycin M_1, a highly sensitive streptogramin antibiotic.

Chemoselective ligation was first developed by protein chemists[1,2] to circumvent the difficulties encountered with conventional solid phase peptide synthesis (SPPS) when making large peptides. The principle is to specifically link two completely unprotected peptides, under mild aqueous conditions, via the chemoselective reaction of a pair of mutually and uniquely reactive functional groups introduced on the peptides during SPPS. Thiol and carbonyl chemistries are extensively used for that purpose. Since its initial development in the protein field, this interesting concept has been extended to the synthesis of numerous groups of molecules, otherwise difficult to obtain, such as cyclic peptides,[3] lipopeptides,[4,5] glycopeptides,[4,6,7] carbohydrate–oligonucleotide conjugates,[8] and steroid derivatives.[9]

Virginiamycin, a streptogramin antibiotic produced by *Streptomyces virginiae*, is composed of two classes of compounds, M and S (for a review see Crooy and De Neys)[10] and inhibits protein synthesis in Gram positive bacteria.[11] Virginiamycin is used as a growth promoting agent in animal feeds, under the commercial name Stafac®. Its therapeutic uses have been limited.[12] Nowadays, the rising number of nosocomial infections has renewed interest in streptogramins. Indeed, dalfopristin, a water-soluble derivative of pristinamycin M_1 factor (virginiamycin M_1) combined with quinupristin, a semi-synthetic derivative of pristinamycin S, is used for the treatment of infections caused by multiple resistant Gram positive bacterial strains (e.g., *Staphylococcus aureus*, *Streptococcus pneumoniae*...).[13] Therefore, a demand for new synthetic routes to access streptogramin derivatives is currently arising. In this paper, we introduce the chemoselective ligation strategy to link amino acids or peptides to virginiamycin M_1.

The M_1 factor (Fig. 1) is the major component of virginiamycin (over 60% by weight); highly purified M_1 (HPLC-UV (214 nm) purity >99%) used throughout this study was obtained as previously described.[14]

In qualitative studies, it has been reported that virginiamycin M factors are subject to degradation reactions in acidic and basic media.[15] In the present study, the stability of purified M_1 factor was monitored by RP-HPLC as

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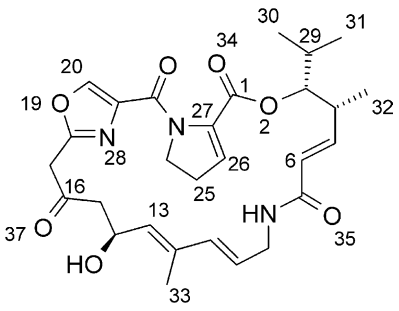


Figure 1. Structure of virginiamycin factor M_1.

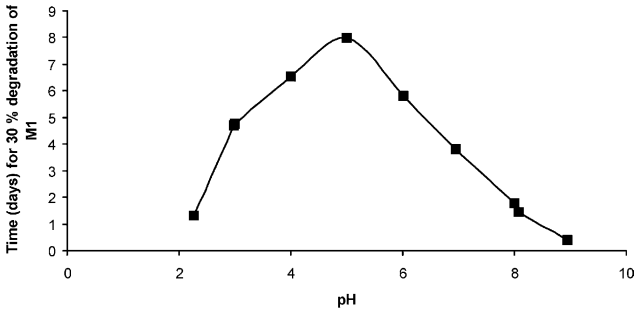


Figure 2. Influence of pH on virginiamycin M_1 stability.

a function of time, using buffers covering the 2–9 pH range. The data presented in Figure 2 confirm pH-mediated modification of M_1, which shows optimal stability at pH 5. This clearly precludes standard Boc or Fmoc protections when linking amino acids or peptides to M_1 through an ester bond involving the hydroxyl group of M_1, which would not resist to the severe deprotection conditions required. Therefore, chemoselective ligation was considered as an alternative and oxime bond formation was chosen as it can selectively involve the ketone function of M_1 within the less disfavourable pH 4–6 range.[16]

Commercially available (Boc-aminooxy)acetic acid (Boc-AOA) was selected to introduce the aminooxy segment on the amino acid moiety. In a preliminary step, in order to check the capability of AOA as connecting arm, unprotected AOA was linked to M_1 via an oxime bond, (AOA.1/2 HCl, pyridine 1.1 equiv, ethanol, 20 h, room temperature) to deliver the expected oxime conjugate M_1AOA. RP-HPLC analysis of the reaction medium confirmed on the one hand that the oxime M_1AOA was produced in almost quantitative yield and on the other hand that both Z and E stereoisomers have been synthesised in a 55:45 ratio. Z and E oximes were efficiently purified and separated by preparative RP-HPLC. After isolation, both stereoisomers were re-injected many times in analytical RP-HPLC to monitor their stability. Figure 3 clearly shows that both Z and E forms undergo isomerisation, leading to a similar equilibrium mixture after at least 10 h. The occurrence of TFA in the eluent increases the oxime isomerisation rate: when purification is performed in the absence of TFA (same column as in Figure 3, isocratic elution H_2O/CH_3CN

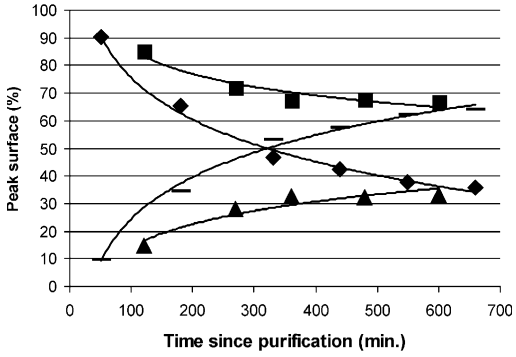


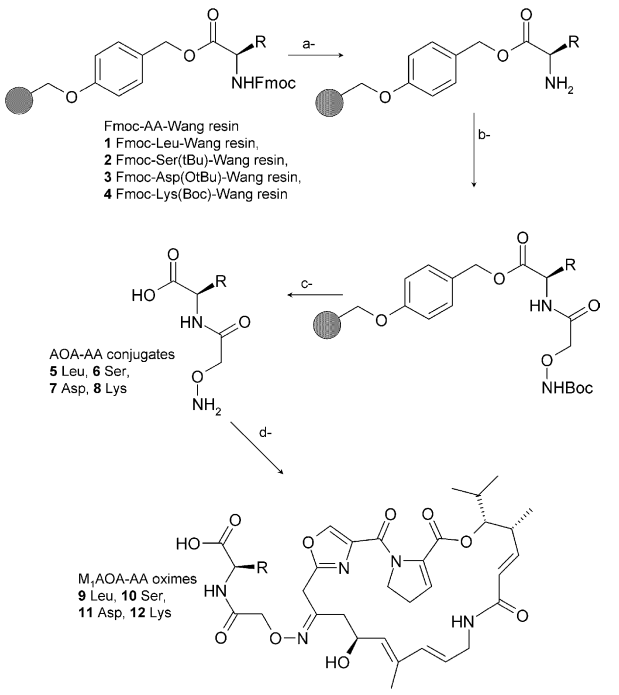
Figure 3. Study of M_1AOA isomerisation. Evolution of the peak surface at 214 nm (RP-HPLC, Vydac, Protein and Peptide C18, 10–15 μm, 250 × 4,6 mm; isocratic elution: $H_2O + 0.05\%$ TFA/$CH_3CN + 0.05\%$ TFA (73:27), 1 mL/min) of each stereoisomer in function of time: (■) % isomer 1 (starting from pure isomer 1); (▲) % isomer 2 (starting from pure isomer 1) and (♦) % isomer 2 (starting from pure isomer 2); (—) % isomer 1 (starting from pure isomer 2). The Z and E stereoisomers of M_1AOA were purified by preparative RP-HPLC (Vydac, Protein and Peptide C18, 10–15 μm, 250 × 22 mm; isocratic elution: $H_2O + 0.05\%$ TFA/$CH_3CN + 0.05\%$ TFA (73:27), 23 mL/min).

(83:17), 23 mL/min), isomerisation still occurs albeit much more slowly, 7 h elapsed before any significative amount of the second isomer appeared.

Connecting Leu, Ser, Asp and Lys to M_1 via oxime bond formation first required introduction of the aminooxy function on amino acid by coupling Boc-AOA via standard Fmoc SPPS using HOBt and TBTU as activating agents. After deprotection and TFA cleavage from the resin, the aminooxy amino acids (**5**–**8**) were freeze-dried, without further purification prior to the reaction with M_1. The chemoselective ligation was undertaken in a mixture of acetonitrile/acetate buffer (0.1 M) at pH 4 (v/v, 1:1) to yield conjugates **9**–**12**. Indeed, according to Cervigni et al.,[4] one volume of acetonitrile can be used to increase the solubility of the products. During the course of this study, it was shown that the use of absolute ethanol led to the same results and this salt-free process was therefore preferred (Scheme 1).

As in the case of M_1AOA, Z/E oxime couples were obtained and purified by preparative RP-HPLC (same column as Figure 3; isocratic elution with H_2O and $CH_3CN + 0{,}05\%$ TFA in a ratio depending on the amino acid linked, 23 mL/min). In contrast with M_1AOA, repeated re-injections in analytical RP-HPLC did not reveal any isomerisation within 24 h, which enabled easy purification of Z and E isomers. This is of particular interest as the biological activity of the virginiamycin derivatives may depend on their configuration. Figure 4 shows the chromatogram of the reaction medium for the synthesis of the M_1AOA–AA conjugate **12** : two major peaks corresponding to the Z/E couple can be observed without any major side product.

The mass spectra of all oximes **9**–**12** were recorded by ESI-MS on a Micromass QT of II (Micromass, Man-



Scheme 1. Synthesis of amino acid derivatives of virginiamycin M_1 by chemoselective oxime ligation: (a) Removal of the N^{α}-Fmoc protecting group: 25% v/v piperidine in *N*-methylpyrrolidone (NMP), rt, 10 min, twice; (b) Coupling of Boc-AOA (3 equiv), 1-hydroxybenzotriazole (HOBt)/N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate (TBTU)/*N*,*N*-diisopropylethylamine (DIEA) (3/2.7/6 equiv), NMP, rt, 60 min, twice; (c) Deprotection of the side chains and cleavage from the resin: 95% v/v trifluoroacetic acid (TFA) in DCM, rt, 75 min; filtration under vacuum; drying under N_2, freeze drying; (d) Oxime synthesis with M_1, 3 equiv AOA-AA, absolute ethanol, rt, 12 h.

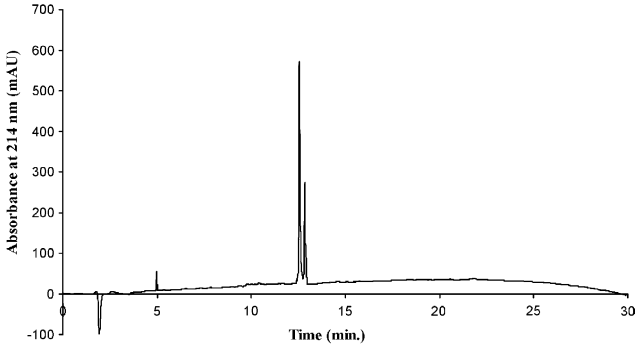


Figure 4. RP-HPLC of M_1AOALys (**12**) reaction medium after 12 h. The negative peak corresponds to the solvent, the small peak at 5 min is the excess AOALys engaged in the reaction and the two major peaks are the Z and E isomer of M_1AOALys. (Agilent, Zorbax 300SB-C18, 3.5 μm, 150 × 4.6 mm; gradient elution: H_2O and CH_3CN + 0.05 % TFA, 0% of organic solvent to 100% in 30 min, 1 mL/min).

chester, UK) spectrometer equipped with an orthogonal electrospray source (Z-spray) operated in positive ion mode. ^1H and ^{13}C NMR spectra were also recorded, using HMBC and HMQC experiments. Comparison of the chemical shifts for virginiamycin M_1[17] and for all synthesised compounds confirmed that every expected oxime conjugates were indeed produced. As an illustration, compared NMR data for M_1 and the separable stereoisomeric lysine conjugates **8a** and **8b** are discussed.[18,19] The ^{13}C shift of the keto group in M_1 is 201.4 ppm: upon oxime formation, C-16 shifts upfield to 152.3 and 153.6 ppm, depending on the configuration. Both α-carbons to oxime also shift upfield: C-17 shifts from 43.7 in M_1 to 28.1 and 34.2 in **8a** and **8b**, respectively, and similarly, C-15 shifts from 49.3 in M_1 to 40.9 and 37.3, respectively. For the β-carbons, a slight downfield shift is observed: +1.0 and +1.1 ppm for C-18; +1.3 and +3.3 for C-14. The above observations are in accordance with the data reported by Hawkes et al.[20] on a large number of ketones and their corresponding oximes. With the exception of H-11, H-13, H-14, H-15 and H-17 (mainly in isomer **12b**), the protons in M_1 conjugates are not significantly affected by oxime formation.

The original chemoselective pathway established within the present study is of particular interest as compared to previously reported works.[21,22] Anteunis et al.[21] made the synthesis of M_1AOATrpOMe by linking Trp methyl ester to the preformed M_1AOA oxime via a classical peptide synthesis strategy. The major drawback of such a procedure was that the final product had to remain protected as no suitable deprotection method was reported. Similarly, using protecting groups Lin et al.[22] linked a tripeptide siderophore to the virginiamycin S_1 factor, which is far more stable than M_1 in acid media.[23] In the present work, irrespective of the side chain of amino acid to be linked to M_1, the chemoselective ligation using oxime formation led to high yields and to the recovery of Z and E isomers which may be highly purified (HPLC-UV (214 nm) purity >99%) by preparative HPLC. The isolation of pure Z and E isomers is of particular concern for structure—biological function relationship studies (underway for the M_1 derivatives synthesised herein) and has very rarely been considered by previous authors studying oximes.

The chemoselective ligation using oxime bond, which is known to be stable both in vitro and in vivo,[24] could be applied to the synthesis of derivatives of many other polyfunctional, very sensitive biologically active natural compounds.

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18. Selected spectral data for M_1AOALys isomer **12a** (R=$(CH_2)_4NH_2$): ^{1}H NMR (500 MHz, DMSO) δ 12.75 (br s, 1H, COOH), 8.47 (s, 1H, H-20 oxazole), 7.89 (d, 1H, $J = 8.0$ Hz, $CONH_C$), 7.80 (dd, 1H, $J = 7.4$ Hz, $J = 4.2$ Hz, NH-8), 7.65 (br s, 2H, NH_2), 6.58 (dd, 1H, $J = 16.0$ Hz, $J = 6.7$ Hz, H-5), 6.26 (t, 1H, $J = 2.9$ Hz, H-26), 5.91 (d, 1H, H-6), 5.83 (d, 1H, $J = 15.8$ Hz, H-11), 5.56 (ddd, 1H, $J = 3.8$ Hz, $J = 6.0$ Hz, H-10), 5.04 (d, 1H, $J = 8.5$ Hz, H-13), 4.76 (dd, 1H, $J = 1.8$ Hz, $J = 9.8$ Hz, H-3), 4.58 (dt, 1H, $J = 8.5$ Hz, $J = 4.6$ Hz, H-14), 4.53 (d, 1H, $J = 15.2$ Hz, H_{Ab}), 4.44 (d, 1H, H_{Ab}), 4.28–4.25 (m, 1H, H_D), 4.22 (d, 1H, $J = 15.6$ Hz, H-17a), 4.01–4.19 (m, 3H, H-9a, H-24), 3.71 (d, 1H, H-17b), 3.67 (br d, 1H, H-9b), 2.67–2.79 (m, 5H, H-4, H-25, H_H), 2.56 (dd, 1H, $J = 14.4$ Hz, H-15a), 2.45 (dr d, 1H, H-15b), 1.91–1.97 (m, 1H, H-29), 1.80–1.74 (m, 1H, H_{Ea}), 1.63–1.68 (m, 4H, H-33, H_{Eb}), 1.56–1.49 (m, 2H, H_G), 1.36–1.30 (m, 2H, H_F), 1.06 (d, 3H, $J = 6.9$ Hz, H-32), 0.95 (d, 3H, $J = 6.7$ Hz, H-30), 0.88 (d, 3H, $J = 6.7$ Hz, H-31). ^{13}C NMR (125 MHz, DMSO) δ 173.2 (COOH), 168.5 (C_BONH), 165.0 (C-7), 160.1 (C-22), 160.0 (C-1), 158.3 (C-18), 152.3 (C-16), 144.9 (C-20), 143.3 (C-5), 136.1 (C-27), 135.2 (C-21), 133.5 (C-13), 133.3 (C-11), 132.6 (C-12), 125.2 (C-10), 125.0 (C-26), 124.8 (C-6), 80.2 (C-3), 72.2 (C_A), 65.0 (C-14), 51.1 (C_D), 50.5 (C-24), 40.9 (C-15), 39.3 (C-9), 38.6 (C_H), 36.6 (C-4), 30.3 (C_E), 29.7 (C-25), 29.1 (C-29), 28.1 (C-17), 26.4 (C_G), 22.2 (C_F), 19.3 (C-30), 18.6 (C-31), 12.7 (C-33), 11.6 (C-32). ESI MS: $m/z = 709$ [M−H_2O+H^+], 727 [M+H^+], 749 [M+Na^+].
19. Selected spectral data for M_1AOALys isomer **12b** (R=$(CH_2)_4NH_2$): ^{1}H NMR (500 MHz, DMSO) δ 8.40 (s, 1H, H-20 oxazole), 7.98 (d, 1H, $J = 8.0$ Hz, CONHc), 7.82 (dd, 1H, $J = 8.2$ Hz, $J = 3.5$ Hz, NH-8), 7.66 (br s, 2H, NH_2), 6.58 (dd, 1H, $J = 16.1$ Hz, $J = 7.6$ Hz, H-5), 6.24 (td, 1H, $J = 2.9$ Hz, H-26), 5.93 (d, 1H, H-6), 5.68 (d, 1H, $J = 16.0$ Hz, H-11), 5.55 (dt, 1H, $J = 4.0$ Hz, $J = 4.0$ Hz, H-10), 4.90 (td, 1H, $J = 9.2$ Hz, $J = 4.5$ Hz, H-14), 4.78 (d, 1H, $J = 1.5$ Hz, $J = 10.0$ Hz, H-3), 4.66 (d, 1H, H-13), 4.53 (s, 2H, H_A), 4.19–4.32 (m, 3H, H_D, H-24), 4.13 (td, 1H, $J = 9.9$ Hz, H-9a), 3.68 (br d, 1H, H-9b), 3.64 (s, 2H, H-17), 2.99 (dd, 1H, H-15a), 2.64–2.86 (m, 5H, H_H, H-4, H-25), 2.27 (dd, 1H, H-15b), 1.93–1.99 (m, 1H, H-29), 1.80–1.73 (m, 1H, H_{Ea}), 1.68–1.60 (m, 1H, H_{Eb}), 1.51–1.56 (m, 5H, H-33, H_G), 1.37–1.37 (m, 2H, H_F), 1.04 (d, 3H, $J = 6.9$ Hz, H-32), 0.95 (d, 3H, $J = 6.7$ Hz, H-30), 0.90 (d, 3H, $J = 6.5$ Hz, H-31). ^{13}C NMR (125 MHz, DMSO) δ 173.2 (COOH), 168.4 (C_BONH), 165.5 (C-7), 160.4 (C-22), 160.3 (C-1), 158.4 (C-18), 153.6 (C-16), 146.4 (C-20), 143.0 (C-5), 136.0 (C-27), 134.9 (C-21), 132.8 (C-12), 132.6 (C-13), 132.2 (C-11), 125.3 (C-10), 125.1 (C-6), 123.4 (C-26), 80.5 (C-3), 72.2 (C_A), 67.0 (C-14), 51.2 (C_D), 50.3 (C-24), 39.0 (C-9), 38.6 (C_H), 37.3 (C-15), 36.7 (C-4), 34.2 (C-17), 30.5 (C_E), 29.8 (C-25), 29.3 (C-29), 26.5 (C_G), 22.3 (C_F), 19.6 (C-30), 19.3 (C-31), 12.4 (C-33), 12.0 (C-32).
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