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Synthesis and Biological Evaluation of a Valinomycin Analog Bearing a Pentafluorophenyl Active Ester Moiety

Lucia D'Accolti,*[,†,‡] Nunzio Denora,[§] Gianluigi La Piana,[∥] Domenico Marzulli,[#] Zuzanna S. Siwy,[⊥] Caterina Fusco,*[,‡] and Cosimo Annese*[,†,‡]

[†]Dipartimento di Chimica, [§]Dipartimento di Farmacia − Scienze del Farmaco, and [∥]Dipartimento di Bioscienze, Biotecnologie e Biofarmaceutica, Università degli Studi di Bari "A. Moro", via Orabona 4, 70126 Bari, Italy

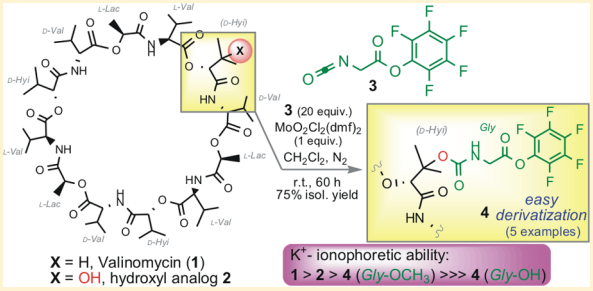
[‡]CNR − Istituto di Chimica dei Composti Organometallici (ICCOM), Bari section, via Orabona 4, 70126 Bari, Italy

[#]CNR − Istituto di Biomembrane e Bioenergetica (IBBE), via Amendola 165/A, 70126 Bari, Italy

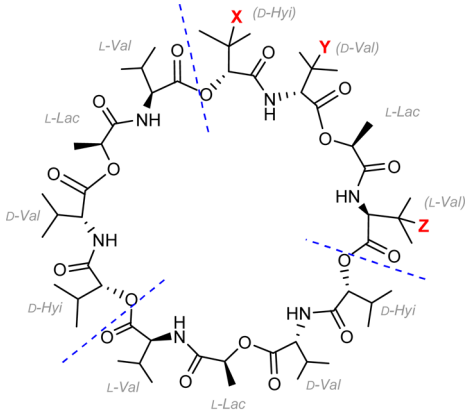
[⊥]School of Physical Sciences, University of California, Irvine, California 92697, United States

Supporting Information

ABSTRACT: A valuable analog of the K^+-ionophore valinomycin (**1**), bearing a pentafluorophenyl ester moiety, has been obtained by selective reaction between the tertiary hydroxyl moiety of analog **2** (available from valinomycin hydroxylation) and the isocyanate group of pentafluorophenyl *N*-carbonyl glycinate (**3**) catalyzed by bis(*N*,*N*-dimethylformamide)dichlorodioxomolybdenum(VI). LC-HRMS studies show that analog **4** undergoes easy derivatization under mild conditions by reaction with OH- and NH_2-containing compounds. Mitochondrial depolarization assays suggest that **4** acts as a K^+-ionophore, provided that the glycine carboxyl group is appropriately masked.



Recently, we have been interested in the antitumor activity of the K^+-ionophore valinomycin (VLM, **1**), a naturally occurring cyclodepsipeptide, chemically consisting of a three repeating sequence of the tetramer D-α-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl (D-Hyi-D-Val-L-Lac-L-Val).[1] The complex $[VLM\text{-}K]^+$ is highly hydrophobic, allowing K^+ to be passively shuttled across membranes. At the level of mitochondria, this causes dissipation of transmembrane potential ($\Delta\Psi_m$) and induction of apoptosis, which has been shown in several mammalian cell types,[2] including a number of tumor cell lines.[3] Aiming at developing VLM-based anticancer drug conjugates, we addressed the issue of providing the macrocycle with a chemical handle (e.g, OH, CO_2H, NH_2) necessary for conjugation. Inspired by the efficiency of the enzyme-catalyzed post-translational modification of peptides occurring in biological systems, we wished to effect a direct modification of VLM, rather than pursuing a typical de novo synthesis of the macrocycle with incorporation of an amino acid residue having a hydroxyl side chain (typically Tyr).[4] Thus, we have described the reaction of VLM with methyl(trifluoromethyl)dioxirane under mild conditions (0 °C, neutral pH) as a valuable synthetic route to VLM analogs bearing an OH handle at the β position of a D-Hyi, D-Val, or L-Val residue (analogs **2**, **2′**, and **2″**, respectively).[5] In spite of this first remarkable achievement, we have become aware that derivatization of tertiary hydroxyl moieties is generally hard to achieve under the mild conditions[6] which are normally required when handling biomolecules like VLM. We could verify, for instance, that carbodiimide-mediated acylation or activation of the OH group with carbonyldiimidazole or triphosgene, according to described procedures,[7,8] give rather unsatisfactory results.



Recently, MoO_2Cl_2 and related complexes have drawn special attention on account of their high versatility, being able to catalyze with high efficiency and under very mild conditions a range of fundamental organic transformations.[9]

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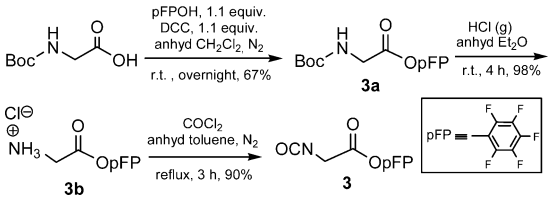
Among other, it has been shown that simple primary, secondary, and even tertiary alcohols can be smoothly converted into their carbamates under mild conditions by reaction with alkyl or aryl isocyanates, in the presence of catalytic amounts of the bis(*N*,*N*-dimethylformamide) complex of MoO_2Cl_2, i.e., $MoO_2Cl_2(dmf)_2$. Despite the broad substrate scope explored, there has been little to no evidence that functional groups other than alkyl or aryl moieties are tolerated during the reaction.[10] Herein, we show that the tertiary hydroxyl moiety of analogs **2**–**2″** can selectively react at the isocyanate functionality of pentafluorophenyl (pFP) *N*-carbonyl glycinate (**3**), under experimental conditions only moderately different from those reported.[10] As a result, a pFP ester moiety can be readily installed on VLM via a carbamate linkage, affording new analogs amenable to easy further derivatization, in view of the high reactivity of pFP esters.

pFP *N*-carbonyl glycinate (**3**) was prepared from Boc-Gly-OH, which was converted into Boc-Gly-OpFP (**3a**) by DCC-mediated esterification with pentafluorophenol (pFPOH). Removal of Boc with HCl(g) in anhydrous Et_2O provided Gly hydrochloride pFP ester (**3b**), which was then converted into pFP *N*-carbonyl glycinate (**3**) by reaction with $COCl_2$ under a N_2 atmosphere (Scheme 1).[11]

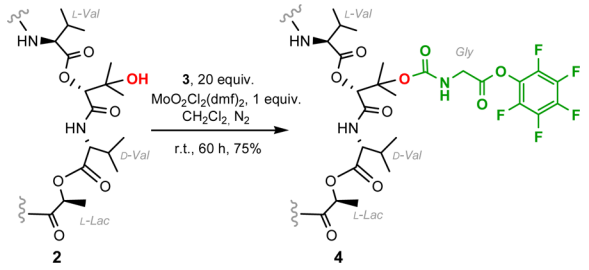
Scheme 1. Synthesis of pFP *N*-Carbonyl Glycinate (3)



Although to a lesser extent than **2′** and **2″**, hydroxyl analog **2** still retains a significant level of VLM bioactivity.[1] On the other hand, it is the major product ensuing VLM hydroxylation.[5] Therefore, such hydroxyl analog was selected as the substrate of choice to carry out all the synthetic and characterization studies presented henceforward.

Preliminary experiments indicated that reaction of analog **2** with isocyanate **3** in the absence of $MoO_2Cl_2(dmf)_2$ results in no products, even in the presence of a large excess of **3** (>20 equiv) and after 72 h reaction. Subsequently, the protocol described by Brückner[10] was applied, and analog **2** (ca. 20 mg) was reacted with 1.2 equiv of isocyanate **3** in the presence of 1% mol $MoO_2Cl_2(dmf)_2$ in anhydrous CH_2Cl_2 (100 μL). Under these conditions, however, only trace amounts of products could be detected by LC-HRMS analysis after 72 h reaction at r.t.. Authors cautioned that successful conversion of sterically congested alcohols into carbamates generally demands an initial concentration of 5 M,[10] which is rather unpractical to realize working on a few tens of μmol of our substrate. Therefore, in a series of subsequent experiments, the amount of isocyanate **3** and of catalyst were gradually increased until 20 equiv and 1 equiv, respectively, at which point complete substrate conversion was observed after 60 h (Scheme 2). Product purification by normal-phase semipreparative HPLC allowed the isolation of the newly VLM analog **4** (75% yield; purity: ≥ 92%, HPLC), the HRMS (ESI) analysis of which was consistent with the expected chemical formula, $C_{63}H_{92}F_5N_7O_{22}$. It is worth mentioning that LC-HRMS analyses of the reaction mixtures of analogs **2′** or **2″** with

Scheme 2. Optimized Reaction of VLM Analog 2 with Isocyanate 3 Catalyzed by $MoO_2Cl_2(dmf)_2$



isocyanate **3** under similar conditions provide signals attributable to the same chemical formula, suggesting that the latter hydroxyl VLMs can be functionalized in the same way as **2**.

1D and 2D NMR spectra offered additional support to the molecular structure assigned to **4**. For instance, notable are the absence of the O*H* resonance (4.52 ppm)[5] in the ^{1}H NMR spectrum of **4** upon derivatization of hydroxyl analog **2** with isocyanate **3** and the appearance of a ^{13}C signal at 156.1 ppm, which can be assigned to the resonance of the carbamate C═O. As depicted in Figure 1, the latter shows a HMBC cross-

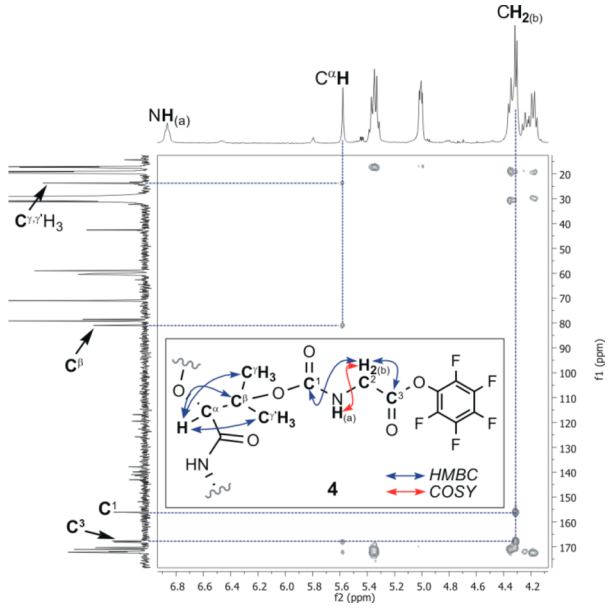


Figure 1. Expansion of the HMBC spectrum and relevant correlations for VLM analog **4**.

peak with a doublet at 4.31 ppm (Gly $C^{\alpha}H_2$), which in turn correlates with a broad ^{1}H signal at 6.87 ppm (Gly N*H*) in the COSY spectrum, and with the pFP ester C═O, located at 167.2 ppm, in the HMBC spectrum. It is also noteworthy that conversion of **2** into **4** causes the resonances of the $C^{\alpha}H$ and of $C^{\gamma,\gamma'}H_3$ of the modified D-Hyi residue to be remarkably downfield-shifted by 0.63, 0.31, and 0.36 ppm, respectively.

Collectively, the spectral data discussed above point out that the hydroxyl group of analog **2** chemoselectively reacts at the isocyanate moiety of the bifunctional building block **3**, leaving the pFP ester one untouched. This appears in line with the previous observation that tertiary alcohols are substantially inert to acylation with catalytic MoO_2Cl_2 at r.t., unless reactions are

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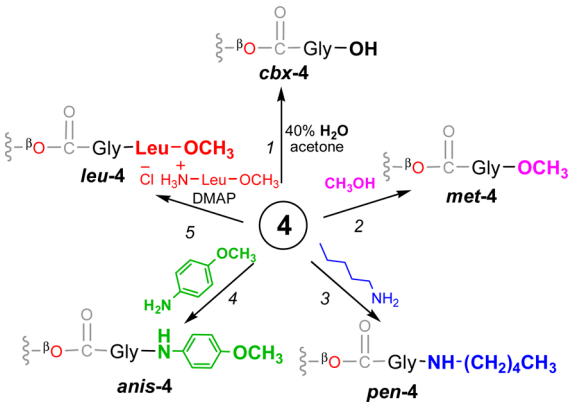
performed at higher temperature and in the presence of a sterically hindered base.[12]

Owing to the high reactivity of pFP esters toward aminolysis, activation of amino acid carboxylic groups as pFP esters has been a major strategy for convenient amide bond formation in peptide synthesis.[13] The easy derivatizability of analog **4** under mild conditions was evaluated by LC-HRMS analysis of reaction mixtures of **4** with representative OH- and NH_2-containing compounds (Scheme 3).

Scheme 3. Derivatization of Analog 4 with Representative OH- and NH_2-Containing Compounds



As shown in Scheme 3, the pFP ester moiety of **4** can be hydrolyzed in 40% aqueous acetone after 60 h (transformation 1) yielding the VLM analog ***cbx*-4** with the unmasked carboxyl group. Similarly, analog **4** can be converted into the methyl ester ***met*-4** within 60 h by dissolution in anhydrous methanol (transformation 2). Expectedly, reaction of **4** with NH_2-containing compounds is considerably faster (transformations 3 and 4): *n*-pentylamine and *p*-methoxyaniline yield the corresponding substitution products ***pen*-4** and ***anis*-4** in a matter of minutes, while aminolysis by Leu methyl ester hydrochloride (transformation 5) requires 3 equiv of DMAP to provide ***leu*-4** in 2 h.

We deemed it useful to evaluate any possible changes in the ionophoretic activity of **2** following attachment of the Gly residue to its hydroxyl side-chain. To this purpose, the dissipation rates of mitochondrial membrane potential ($\Delta\Psi_m$)[14] induced by analogs **2**, ***met*-4**, ***cbx*-4**, and the unmodified macrocycle **1** (control) were compared. ***met*-4** was used as a substituted of the more labile **4**, and its ionophoretic activity compared to that of ***cbx*-4**, in which the unmasked carboxyl group makes it the higher polar extreme. Experiments were performed on isolated rat-liver mitochondria suspended in a 125 mM KCl medium to closely mimic the physiological [K^+] inside cells. The mitochondrial suspension was added with increasing concentrations of each macrocycle, and the $\Delta\Psi_m$ decay was monitored fluorimetrically, according to established protocols.[14,15] Results are graphically summarized in Figure 2.

As shown in Figure 2, $\Delta\Psi_m$ depolarization rate values, expressed as fluorescence units/min, indicate that VLM (**1**) is expectedly the most effective depolarizing agent. When compared in terms of the concentrations required to yield a given $\Delta\Psi_m$ dissipation rate value, the efficiency of **1**, **2**, ***met*-4**, and ***cbx*-4** as ionophores decreases in the order: **1** > **2** > ***met*-4** ≫ ***cbx*-4**. In particular, ***met*-4** requires a ca. 2.5-fold higher

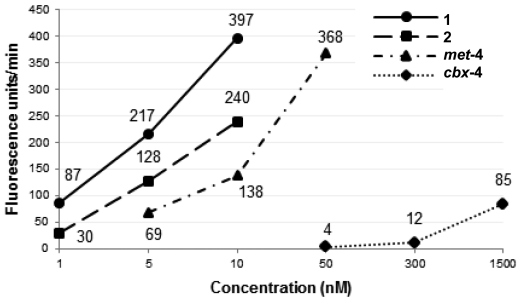


Figure 2. Rates of $\Delta\Psi_m$ decay (fluorescence units/min) induced by increasing concentrations of **1**, **2**, ***met*-4**, and ***cbx*-4**. Experiments were performed on isolated rat-liver mitochondria suspended in a 125 mM KCl medium. Estimated rates are average values from three independent runs (RSD 4–6%).

concentration to collapse $\Delta\Psi_m$ at the same rate of its parent hydroxyl analog **2**, while ***cbx*-4** appears barely active, since its concentration should be approximately 500 times higher than that of **2** to collapse $\Delta\Psi_m$ at the same rate.

It should be recalled that a number of kinetics and thermodynamic factors underlie the efficacy of molecules of the group of VLM to function as ionophores, high stability and sufficient lipophilicity of the K^+ complex, to name a few.[16] The finding that ***met*-4** and ***cbx*-4** retain the K^+-complex properties of VLM[17] is in line with the claim that modification of VLM side chains does not introduce sizable variations in its K^+-complex properties.[14,16] Owing to the presence of the highly polar carboxylic group, it can be argued that ***cbx*-4** cannot meet the "sufficient lipophilicity" criterion mentioned previously to establish a transmembrane K^+ flux able to efficiently collapse $\Delta\Psi_m$.

In summary, reaction of the VLM hydroxyl analogs **2**–**2″** with pFP *N*-carbonyl glycinate (**3**) catalyzed by $MoO_2Cl_2(dmf)_2$, under mild conditions, offers a convenient way to access VLM analogs bearing a Gly pFP ester moiety, as exemplified by the isolation and full spectroscopic characterization of analog **4**. Mitochondrial depolarization assays suggest that, upon attachment of the Gly residue to the OH appendage of **2**, the ionophoretic activity of the macrocycle is only moderately reduced, provided that the Gly carboxyl group is appropriately masked. The newly VLM analog **4** is expected to serve well in a number of applications, primarily in the field of medicinal and materials chemistry,[18] as suggested by the ease with which the pFP ester moiety undergoes derivatization. It should be emphasized that, unlike de novo synthetic routes to derivatizable VLM analogs,[4] the synthesis of **4** described herein merely involves a selective chemical modification of the native VLM, witnessing the high level of efficiency reached by modern chemical processes, which in several cases compare well with those developed by Nature. In this instance, the present study attests to the high potential of the isocyanate/MoO_2Cl_2 system in targeting naturally occurring substrates, as this carbamylation protocol requires mild experimental conditions and tolerates sensitive functional groups and delicate substrates.

EXPERIMENTAL SECTION

General Experimental Methods. The ^1H NMR spectra were recorded at 500 MHz and referenced to residual isotopic impurity of chloroform-*d* (7.26 ppm) or of acetone-d_6 solvent (2.05 ppm). The $^{13}C\{^1H\}$ NMR spectra were recorded at 125 MHz and referenced to the middle peak of chloroform-*d* (77.00 ppm) or of acetone-d_6 solvent (29.84 ppm). The ^{19}F NMR spectra were recorded at 470 MHz and

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referenced to the $CFCl_3$ signal (0.00 ppm), used as an internal standard. GC-MS experiments were run using a J&W DB-5 ms column (30 m × 0.25 mm id, film thickness 0.25 μm). High-resolution mass spectra (ESI-TOF) were recorded with the electrospray ion source in positive-ion mode; the following instrument settings were used: nebulizer gas, nitrogen at 1.5 L/min; dry gas, nitrogen at 102 MPa and 250 °C; collision gas, argon. The LC-HRMS analyses were carried out using a Eurospher II 100−3 C18 column (100 × 2 mm ID); conditions: water (A)−methanol (B) mobile phase; gradient elution, 90−100% B in 10 min, 0.15 mL/min. Unless noted otherwise, semipreparative HPLC separations were carried out in normal phase by employing an Ascentis, Si coulmn (250 × 10 mm, 5 μm); elution conditions: isocratic 3:1 diethyl ether/hexane, flow rate 2.5 mL/min, UV detector 220, 254 nm. *Cyclo*[(D-α-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl)$_2$(D-α,β-dihydroxyisovaleryl-D-valyl-L-lactyl-L-valyl)] (analog **2**) was prepared from commercially available valinomycin (**1**), following our previously described procedure.[5] *N*-Boc-glycine pentafluorophenyl ester (**3a**) was obtained from commercial Boc-Gly-OH upon esterification with pentafluorophenol, following a described procedure.[19] Bis(*N*,*N*-dimethylformamide)dichlorodioxomoybdenum(VI), $MoO_2Cl_2(dmf)_2$ was obtained from commercial dichlorodioxomoybdenum(VI), MoO_2Cl_2, according to a reported procedure.[20]

*Pentafluorophenyl N-Carbonyl Glycinate (**3**).* Dry HCl(g) was bubbled into a stirred solution of Boc-Gly-OpFP **3a** (0.40 g, 1.17 mmol) in anhydrous diethyl ether (20 mL), kept at 0 °C, for ca. 30 min. Stirring was continued at r.t. until TLC analysis (silica gel, eluent CH_2Cl_2) revealed complete substrate consumption (4 h). The white solid that precipitated during the reaction was collected by filtration, washed with anhydrous diethyl ether, and dried under vacuum, providing 0.32 g (1.15 mmol, yield 98%) of glycine pentafluorophenyl ester hydrochloride (**3b**); HRMS (ESI-TOF) *m*/*z*: $[M]^+$ Calcd for $C_8H_5F_5NO_2^+$ 242.0235; Found 242.0255. Under a N_2 atmosphere, a three-necked flask was charged with freshly prepared glycine pentafluorophenyl ester hydrochloride **3b** (0.29 g, 1.05 mmol) and anhydrous toluene (10 mL). The resulting suspension was then heated at reflux. A neck of the flask was connected to a refrigerant kept at −10 °C, while a glass needle, dipping into the reaction suspension, was jointed to the other neck. The glass needle was connected by a tube to a Schlenk tube surmounted by a funnel for addition of solids charged with solid triphosgene. The Schlenk tube contained 1 mg of Cu-phtalocyanine in 2 mL of anhydrous toluene at 90 °C. Small portions of triphosgene were added to the Schlenk tube every 10 min, allowing a gentle stream of $COCl_2$ to bubble into the reaction flask until glycine pentafluorophenyl ester hydrochloride (**3b**) dissolved, and the reaction mixture turned clear (ca. 3 h). Then, the reaction mixture was cooled to r.t., and the excess $COCl_2$ and HCl removed from the reaction flask by a stream of N_2 (collecting the outflowing gas into a flask containing ethanol). The resulting solution was filtered under N_2, to remove traces of unreacted substrate, and concentrated under reduced pressure to provide pentafluorophenyl *N*-carbonyl glycinate (**3**, 0.25 g, 0.94 mmol, yield 90%, purity ≥98% by ^{1}H NMR) as a white solid, mp 48.5−49.2 °C; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.38 (s, CH_2) ppm; $^{13}C\{^1H\}$ NMR ($CDCl_3$, 125 MHz): δ 165.7, 140.9 (m, $^1J_{CF}$ = 251 Hz, CF), 140.0 (m, $^1J_{CF}$ = 254 Hz, CF), 137.9 (m, $^1J_{CF}$ = 252 Hz, CF), 126.8 (N═C═O), 124.4 (pFP CO), 43.7 ppm; ^{19}F NMR ($CDCl_3$, 470 MHz): δ −152.8 (d, *J* = 17 Hz, 2F, *ortho*-F), −156.8 (t, *J* = 22 Hz, 1F, *para*-F), −162.4 (dd, J_1 = 22, J_2 = 17 Hz, *meta*-F) ppm; FT-IR (KBr): ν 2259 cm^{-1}; GC-MS (EI, 70 eV) *m*/*z* (r.i.): 267 (M^+, 0.5), 239 (M^+ − CO, 8.6), 184 (pFPOH$^+$, 2), 56 ($OCNCH_2^+$, 100); HRMS (EI) *m*/*z*: $[M]^+$ Calcd for $C_9H_2F_5NO_3^+$ 266.99548; Found 266.99414.

*Cyclo[(D-α-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl)$_2$(D-α-hydroxy-β-(oxycarbonylglycine pentafluorophenyl ester)isovaleryl-D-valyl-L-lactyl-L-valyl)] (**4**).* N_2 was flowed through a 5 mL round-bottomed flask by means of a stainless-steel needle inserted into a septum. Analog **2** (21 mg, 18.6 μmol), pentafluorophenyl *N*-carbonyl glycinate (**3**, 99.4 mg, 372 μmol, 20 equiv), and $MoO_2Cl_2(dmf)_2$ (6.4 mg, 18.6 μmol) were sequentially added, and the septum secured to the flask. Under stirring, 100 μL of anhydrous CH_2Cl_2 were added by means of a syringe, and stirring was continued at r.t. until TLC analysis (silica gel, eluent diethyl ether/hexane 3:1, iodine/1% aqueous starch solution) revealed complete substrate consumption (60 h). After evaporating CH_2Cl_2 by a stream of N_2, the crude product was diluted with anhydrous diethyl ether, filtered through a silica gel pad, and concentrated under reduced pressure. Purification by semipreparative normal-phase HPLC afforded 19.5 mg of **4** (14.0 μmol, yield 75%, purity ≥92% by HPLC); ^{1}H NMR (acetone-d_6, 500 MHz): δ 7.97−7.93 (m, 3H, N*H*), 7.70 (d, *J* = 8.2 Hz, 1H, N*H*), 7.67 (d, *J* = 7.9 Hz, 1H, N*H*), 7.63 (d, *J* = 7.4 Hz, 1H, N*H*), 6.87 (br m, 1H, Gly N*H*), 5.58 (s, 1H, D-$^{\beta}$GlyHyi C$^{\alpha}$*H*), 5.39−5.32 (m, 3H, L-Lac C$^{\alpha}$*H*), 5.01 (d, *J* = 7.7 Hz, 1H, D-Hyi C$^{\alpha}$*H*), 5.00 (d, *J* = 7.5 Hz, 1H, D-Hyi C$^{\alpha}$*H*), 4.35 (m, 3H, Val C$^{\alpha}$*H*), 4.31 (d, *J* = 5.5 Hz, 2H, Gly C$^{\alpha}$*H*$_2$), 4.24 (m, 1H, Val C$^{\alpha}$*H*), 4.21−4.16 (m, 2H, Val C$^{\alpha}$*H*), 2.37−2.33 (m, 5H, D-Hyi and Val C$^{\beta}$*H*), 2.27−2.18 (m, 3H, Val C$^{\beta}$*H*), 1.63 (s, 3H, D-$^{\beta}$GlyHyi C$^{\gamma}$*H*$_3$), 1.55 (s, 3H, D-$^{\beta}$GlyHyi C$^{\gamma'}$*H*$_3$), 1.42−1.40 (m, 9H, L-Lac C$^{\beta}$*H*$_3$), 1.09−0.97 (m, 48H, Val and D-Hyi C$^{\gamma,\gamma'}$*H*$_3$) ppm; $^{13}C\{^1H\}$ NMR (acetone-d_6, 125 MHz): δ 172.4, 172.33, 172.26, 172.24, 172.18, 171.9, 171.12, 171.08, 171.0, 170.4, 170.3, 168.2, 167.2 (Gly C═O), 156.1 (carbamate C═O), 142.1 (m, $^1J_{CF}$ = 253 Hz, CF), 140.5 (m, $^1J_{CF}$ = 248 Hz, CF), 138.9 (m, $^1J_{CF}$ = 249 Hz, CF), 125.8 (pFP CO), 81.0 (D-$^{\beta}$GlyHyi C$^{\beta}$), 79.2 (D-Hyi C$^{\alpha}$H), 78.5 (D-$^{\beta}$GlyHyi C$^{\alpha}$H), 71.1 and 71.0 (L-Lac C$^{\alpha}$H), 60.5, 60.3, 60.0, 59.1, 59.0, and 58.97 (Val C$^{\alpha}$H), 42.6 (Gly C$^{\alpha}$H$_2$), 31.21, 31.19, and 30.6 (D-Hyi and Val C$^{\beta}$H), 23.8 (D-$^{\beta}$GlyHyi C$^{\gamma'}$H$_3$), 23.7 (D-$^{\beta}$GlyHyi C$^{\gamma'}$H$_3$), 19.72, 19.70, 19.63, 19.60, 19.53, 19.44, 19.41, 19.35, 19.24, 19.1, 19.02, 19.00, 17.43, 17.40, 17.3, and 17.1 (L-Lac C$^{\beta}$H$_3$ and Val, D-Hyi C$^{\gamma,\gamma'}$H$_3$) ppm; ^{19}F NMR (acetone-d_6, 470 MHz): δ −153.51 (d, *J* = 17 Hz, 2F, *ortho*-F), −159.15 (t, *J* = 21 Hz, 1F, *para*-F) −163.63 (dd, J_1 = 21 Hz, J_2 = 17 Hz, 2F, *meta*-F) ppm; HRMS (ESI-TOF) *m*/*z*: $[M + K]^+$ Calcd for $C_{63}H_{92}F_5N_7O_{22}K^+$ 1432.5847; Found 1432.5856.

LC-HRMS Studies on Analog 4 Derivatization. Transformations 1 and 2: 0.5 mg of **4** was dissolved in 1 mL of the appropriate solvent (40% aqueous acetone for 1 and anhydrous CH_3OH for 2), and the solution left standing at r.t. until the LC-HRMS analysis revealed that **4** had consumed (60 h); ***cbx*-4**: HRMS (ESI-TOF) *m*/*z*: $[M + NH_4]^+$ Calcd for $C_{57}H_{97}N_8O_{22}^+$ 1245.6717; Found 1245.6714; ***met*-4**: HRMS (ESI-TOF) *m*/*z*: $[M + NH_4]^+$ Calcd for $C_{58}H_{99}N_8O_{22}^+$ 1259.6874; Found 1259.6870.

Transformations 3 and 4: 0.5 mL of a 0.72 mM stock solution of **4** in anhydrous CH_2Cl_2 was mixed with 50 μL of a 11 mM solution of *n*-pentylamine (for 3) or *p*-methoxyaniline (for 4) in anhydrous CH_2Cl_2. LC-HRMS analysis of the mixture revealed consumption of the substrate in a few minutes; ***pen*-4**: HRMS (ESI-TOF) *m*/*z*: $[M + NH_4]^+$ Calcd for $C_{62}H_{108}N_9O_{21}^+$ 1314.7660; Found 1314.7624; ***anis*-4**: HRMS (ESI-TOF) *m*/*z*: $[M + NH_4]^+$ Calcd for $C_{64}H_{104}N_9O_{22}^+$ 1350.7296; Found 1350.7282.

Transformation 5: To 1 mL of a 0.72 mM stock solution of **4** in anhydrous CH_2Cl_2, 0.2 mg (1.5 equiv) of leucine methyl ester hydrochloride, followed by 0.26 mg (3 equiv) of DMAP were added. LC-HRMS analysis of the mixture revealed consumption of the substrate within 2 h; ***leu*-4**: HRMS (ESI-TOF) *m*/*z*: $[M + NH_4]^+$ Calcd for $C_{64}H_{110}N_9O_{23}^+$ 1372.7715; Found 1372.7733.

Mitochondrial Depolarization Assays. ***cbx*-4** and ***met*-4**, obtained as described above, were purified by semipreparative HPLC (column: Supelcosil LC-18, 250 × 10 mm ID, 5 μm; conditions: isocratic elution methanol−water 9:1, UV detector 220 nm). The collected fractions, concentrated under reduced pressure and then lyophilized, contained each product with purity ≥94% (HPLC). Rat-liver mitochondria were isolated in mannitol-sucrose medium as previously described,[14] and the oxidative phosphorylation efficiency was assayed by measuring the respiratory control index.[14] Incubations were carried out at 25 °C in a standard medium containing 125 mM KCl and 20 mM HEPES-Tris, pH 7.4. $\Delta\Psi_m$ variations were followed fluorimetrically, according to the safranine O method,[14] with the spectrofluorimeter set at the excitation and emission wavelengths of 520 and 580 nm, respectively. Typically, to a cuvette containing 3.0 mL of the standard medium, rat-liver mitochondria (1 mg/mL), 6 μM rotenone, and 10 μM safranine O were added. After equilibration, required to dissipate the $\Delta\Psi_m$

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generated by endogenous substrates, the instrument was zeroed, and 5 mM Tris-Succinate was added, followed by either **1**, **2**, ***met*-4**, or ***cbx*-4** (ethanol solutions) to the desired concentration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b02219.

NMR spectra of **3** (^{1}H, ^{13}C, ^{19}F) and **4** (^{1}H, ^{13}C, ^{19}F, COSY, HSQC, HMBC); LC-HRMS data for **4**, ***cbx*-4**, ***met*-4**, ***anis*-4**, ***pen*-4**, and ***leu*-4**; HPLC chromatograms of samples of ***cbx*-4** and ***met*-4**; selected curves of $\Delta\Psi_m$ decay (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Email: lucia.daccolti@uniba.it.
*Email: fusco@ba.iccom.cnr.it.
*Email: annese@ba.iccom.cnr.it.

Notes

The authors declare no competing financial interest.

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