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Inhibitors of HCV NS5A: From Iminothiazolidinones to Symmetrical Stilbenes

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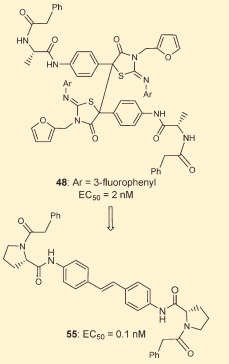
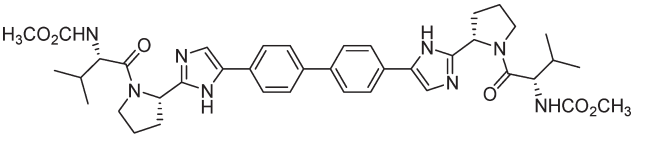
Supporting Information

ABSTRACT: The iminothiazolidinone BMS-858 (**2**) was identified as a specific inhibitor of HCV replication in a genotype 1b replicon assay *via* a high-throughput screening campaign. A more potent analogue, BMS-824 (**18**), was used in resistance mapping studies, which revealed that inhibitory activity was related to disrupting the function of the HCV nonstructural protein 5A. Despite the development of coherent and interpretable SAR, it was subsequently discovered that in DMSO **18** underwent an oxidation and structural rearrangement to afford the thiohydantoin **47**, a compound with reduced HCV inhibitory activity. However, HPLC bioassay fractionation studies performed after incubation of **18** in assay media led to the identification of fractions containing a dimeric species **48** that exhibited potent antiviral activity. Excision of the key elements hypothesized to be responsible for antiviral activity based on SAR observations reduced **48** to a simplified, symmetrical, pharmacophore realized most effectively with the stilbene **55**, a compound that demonstrated potent inhibition of HCV in a genotype 1b replicon with an EC_{50} = 86 pM.

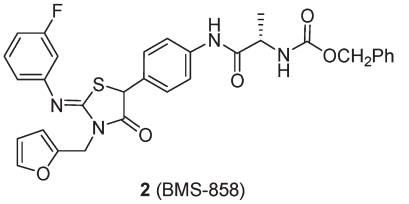


KEYWORDS: HCV NS5A inhibitor, iminothiazolidinone, stilbene

Almost 200 million individuals worldwide are infected with hepatitis C virus (HCV), a disease for which the current, optimal therapy consists of weekly subcutaneous injections of pegylated interferon-α (PEG-IFN) in combination with twice-daily oral doses of ribavirin (RBV).[1−3] The inadequacies of this treatment regimen are evident in the low response rates for genotype 1-infected patients and the significant incidence of side effects. The development of direct acting antiviral agents (DAAs) to treat HCV has focused primarily on inhibitors of NS3 protease and the NS5B RNA-dependent RNA polymerase, and several compounds are presently in clinical trials as add-on to PEG-IFN/RBV therapy.[4] We have recently reported the discovery of the HCV NS5A inhibitor BMS-790052 (**1**), a compound that demonstrates a potent antiviral effect in HCV genotype 1-infected subjects following single oral doses.[5] In this article we describe the preliminary structure−activity relationships (SAR) developed for the HTS screening hit **2** (BMS-858) and delineate a chemical reactivity inherent to this class of compound that led to the elucidation of a structurally complex dimeric NS5A inhibitor from which a simplified, symmetrical pharmacophore was excised as a prelude to the design of BMS-790052 (**1**).



1 (BMS-790052)



2 (BMS-858)

Using a dual HCV genotype 1b/bovine viral diarrhea virus replicon screen in high throughput mode, the thiazolidinone **2**

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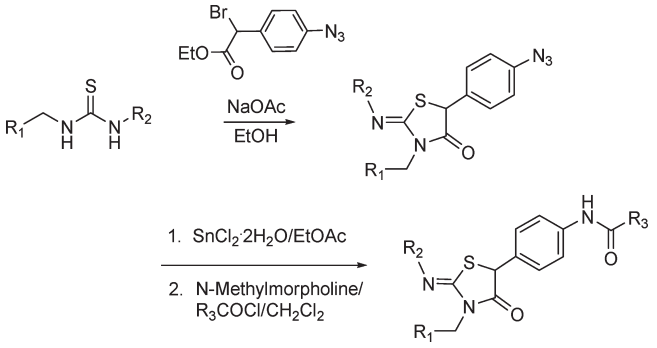
Scheme 1. Synthetic Approach to Iminothiazolidinones Using Parallel Solution Phase Chemistry

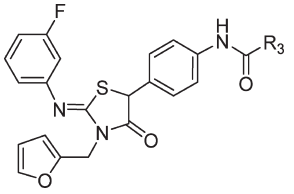


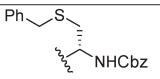
was identified as a potent HCV inhibitor, EC_{50} = 0.58 μM, that demonstrated a good therapeutic window with respect to cytotoxicity (CC_{50} = >100 μM) and showed no significant inhibitory activity in counterscreens.[6,7] This compound had been prepared as part of a prospective library according to the synthetic route depicted in Scheme 1, where regiocontrol during ring closure occurred as anticipated based on literature precedent.[8−10] Assignment of the 2-arylimino *Z*-configuration is also based on prior studies, a function of minimizing unfavorable steric interactions,[11,12] while the C-5 position is configurationally unstable and cannot be defined.[13] Screening results for additional library members were available (see compounds **3**−**7**) and gave indication that antiviral activity was closely associated with the embedded amino acid moiety (Table 1).[7] To expand upon this observation, a survey of amino acid analogues was conducted that confirmed that the L-configuration was essential for antiviral activity since the unnatural, D-configured **8** was over 150-fold weaker, an observation reproduced in the proline derivatives **9** and **10.** Lastly, the data obtained for compounds **12**−**15** communicate a clear preference for smaller Cα substituents.[14]

Based on the results obtained for **2** and **9** in Table 1, L-alanine and L-proline were selected as substrates to probe the SAR at the carboxybenyl (Cbz) terminus (R_3), the 3-fluorophenyl ring (R_2), and furan heterocycle (R_1), as collated in Tables 2 and 3. Exchange of the Cbz for either benzoyl (**16** and **17**) or the isosteric phenylpropionoyl (**20** and **21**) moieties resulted in reduced potency, whereas a phenacetyl proved to be optimal in both the alanine **18** and proline **19** analogues, enhancing potency by ∼100-fold compared to the cases of **2** and **9**, respectively. The high level of potency observed for **18** (BMS-824), EC_{50} = 0.006 μM, facilitated its use in resistance mapping studies designed to illuminate aspects of the mode of antiviral activity. These studies implicated the NS5A protein as the target of this class of inhibitor based on the identification of a Y93H change that, when introduced into the replicon, significantly compromised the potency of **18**, EC_{50} > 5 μM.[5,15]

The parallel SAR observed between the two amino acid series extended to variation of substituents attached to the iminothiazolidinone ring, as shown in Table 3. For example, 2-pyridyl and 3-pyridyl heterocycles in place of the furan enhanced potency by >15-fold compared to the case of **2** in both the alanine (**23** and **25**) and proline (**24** and **26**) series, whereas the more lipophilic 2,3,4-trifluorophenyl ring merely preserved potency (**28** and **29**). Somewhat analogously, lipophilic substituents appended to the 3-phenyl ring (compounds **30**−**37**) generally resulted in reduced antiviral activity compared to the case of **2**, while more

Table 1. Structure−Activity Relationships Associated with Thiazolidinones as Inhibitors of HCV 1b Replicon Activity



Compound #	R_3	EC_{50} (μM)[a]
2 (BMS-858)		0.58
3	OCH_2Ph	150
4	CH_2OCH_2Ph	61
5	$CH(CH_3)OCH_2Ph$	132
6	CH_2CH_2Ph	75
7	$(CH_2)_3CO_2CH_3$	150
8		96
9		0.11
10		6.5
11		5
12		>50
13		6
14		>50
15		>50

[a] EC_{50} values are averages of at least two independent determinations.

polar substituents were beneficial in improving potency, as illustrated by compounds **38**−**45**.

Although the data reported in Tables 1−3 provide a coherent and interpretable SAR, we subsequently discovered C-5 phenyliminothiazolidinones **2** and **18** were consumed during the course of the 3 day replicon assay. The first indication of an inherent chemical reactivity emerged when it was observed that, on standing in DMSO, compound **2** underwent an oxidative rearrangement to generate the thiohydantoin **46** (Scheme 2).[16,17] The structure of **46** was elucidated by correlation spectroscopy, which established the proximity of the furanyl-methylene protons (δ5.2, dd, 2H) to the amide carbonyl carbon (δ 171.4 ppm) and the thiourea carbon (δ 182.5 ppm) atoms. In addition,

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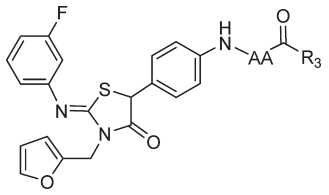
nuclear Overhauser effects (nOe) were observed between the *ortho* protons on the two phenyl rings at δ 9.09 (d, 1H) and δ 7.30 (d, 2H), as depicted in structure **46**.[16]

Thiohydantoin **46**, however, exhibited poor activity in the replicon assay, with an EC_{50} = 13 μM, suggesting that other species in the cell culture media may be responsible for the observed inhibitory activity of **2**, and this prompted a more detailed analysis of compound **18**.[17] While stable indefinitely in solid form and quite stable in solvents such as MeOH and CH_3CN, the iminothiazolidinone **18** underwent the same oxidative transformation in DMSO as **2**, to form **47** (EC_{50} > 20 μM).[16] Moreover, preincubation of **18** in assay media until completely consumed, as determined by HPLC, followed by analysis of the replicon inhibitory activity of the solution, produced an equivalent antiviral effect to that recorded for parent compound **18** under standard assay conditions. HPLC analysis of the preincubated media detected the thiohydantoin **47** as the predominant product; however, it was observed to undergo degradation over time to the thiourea starting material depicted in Scheme 1, presumably *via* hydrolysis of intermediate **C** (Scheme 2). As a result of these findings, HPLC biogram fractionation experiments were performed, an exercise that traced potent HCV inhibitory activity to two minor components in the media identified as isomers of the dimeric species **48** (Figure 1).[17] The connectivity of the dimer was established by ^{1}H and ^{13}C NMR to be at the C-5 methine carbon, with the less mobile isomer on reversed phase LC analysis being the more potent compound, EC_{50} = 0.6 nM, and which converted to the more mobile but less potent (EC_{50} = 43 nM) isomer upon heating at 55 °C.[17]

In considering how compounds **46**−**48** might be generated, it was recognized that hydrogen abstraction at the C-5 methine carbon of **18** would form a classic captodative radical stabilized by the electron donating sulfur atom and the electron withdrawing carbonyl group.[18,19] A radical mechanism is consistent with the formation of **47** by a process involving combination of a C-5 radical with oxygen that would give hydroperoxide **A** (Scheme 2). Precedent for peroxide formation and reductive cleavage in/by

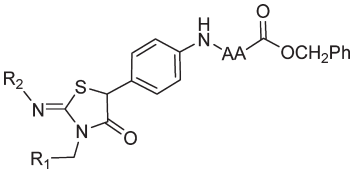
Table 2. Comparison of the Effect of Structural Variation of the Amino Acid N Substituent on Replicon Potency in the L-Alanine and L-Proline Series



		AA = L-Alanine		AA = L-Proline
R_3	cmpd	EC_{50} (μM)[a]	cmpd	EC_{50} (μM)[a]
Ph	**16**	1.9	**17**	7.9
CH_2Ph	**18** (BMS-824)	0.006	**19**	0.023
CH_2CH_2Ph	**20**	3.1	**21**	5.1

[a] EC_{50} values are averages of at least two independent determinations.

Table 3. Effect of Variation of the 2-Furyl and 3-Fluorophenyl Moieties in Both the L-Alanine and L-Proline Series on Antiviral Activity in a HCV Genotype 1b Replicon



R_1	R_2	cmpd	AA = L-alanine EC_{50} (μM)[a]	cmpd	AA = L-proline EC_{50} (μM)[a]
2-tetrahydrofuranyl	3-F-C_6H_4	**22**	0.65		
2-pyrazinyl	3-F-C_6H_4	**23**	0.025	**24**	0.038
2-pyridinyl	3-F-C_6H_4	**25**	0.035	**26**	0.002
3-pyridinyl	3-F-C_6H_4	**27**	0.026		
2,3,4-F_3-C_6H_2	3-F-C_6H_4	**28**	0.55	**29**	0.6
2-furyl	4-CF_3-C_6H_4	**30**	2.2	**31**	0.98
2-furyl	3-F-4-CF_3-C_6H_3	**32**	2.5	**33**	2.2
2-furyl	4−Cl-C_6H_4	**34**	0.51	**35**	1.17
2-furyl	4-Et-C_6H_4	**36**	1.5	**37**	1.8
2-furyl	4-CN-C_6H_4	**38**	0.037	**39**	0.086
2-furyl		**40**	0.01	**41**	0.009
2-furyl	5-quinolinyl	**42**	0.02	**43**	0.01
2-furyl	4-morpholinylphenyl	**44**	0.015	**45**	0.02

[a] EC_{50} values are averages of at least two independent determinations.

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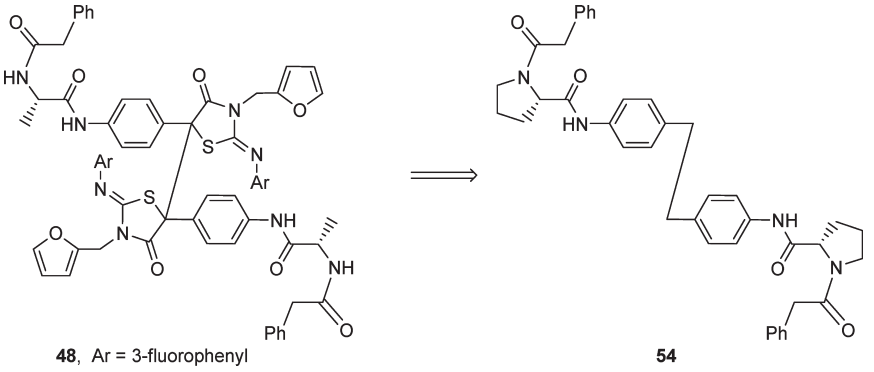
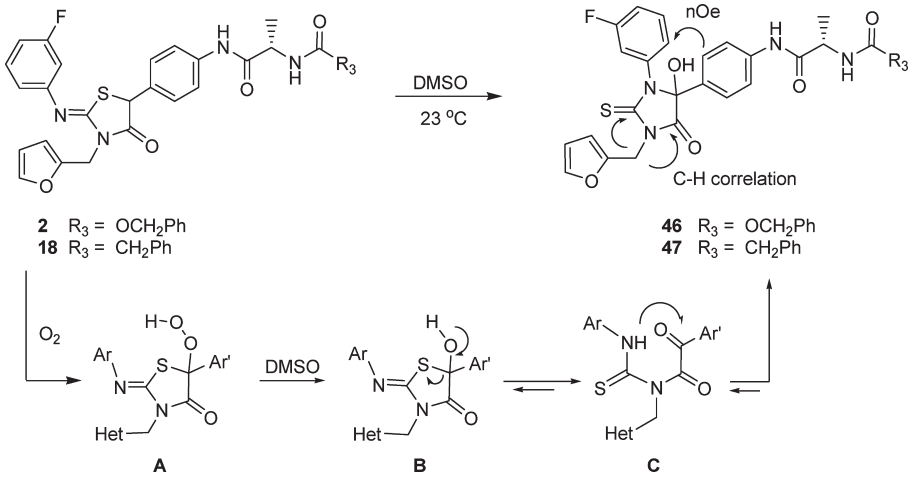


Figure 1. Dimeric species identified in replicon assay media and pharmacophore hypothesis.

Scheme 2. Oxidative Rearrangement of the Iminothiazolidinones 2 and 18



DMSO, which in this case affords **B**, is available in the literature for other systems.[20,21] Unique to **B**, however, is the potential for ring-opening to the α-ketoamide **C**, which can reclose in a complementary fashion to form the 2-thiohydantoin **47**, with experimental support for this sequence of events obtained as follows. Snider has reported that radicals generated in the presence of $Mn(OAc)_3/Cu(OAc)_2$ can undergo further oxidation in which the resulting cation is trapped by acetic acid.[22,23] The phenyl acetamide **49** was exposed to these conditions (Scheme 3) and gave **50**, an isolable derivative of **B**, in 59% isolated yield together with some of the rearranged thiohydantoin **51** in 16% yield. The structure of compound **50** was established by N−H long-range NMR correlation in order to rule out the acetoxy derivative of **51**, which may result simply from exchange of the hydroxyl group with acetic acid.[24]

Exposure of **2** and **18** to the same reaction conditions gave the acetoxy derivatives **52** (48%) and **53** (78%), respectively, with no evidence of thiohydantoin formation. In the latter case, a small amount of dimeric product **48** was obtained (12 mg, 2.3%), which was shown by ^{1}H NMR and LC to be identical with the more mobile dimeric isomer isolated from media.[25,26] In a further experiment, acetates **52** and **53** were subjected to basic methanol (K_2CO_3/MeOH), which effected rapid conversion to thiohydantoins **46** and **47**.[27]

Taken together, these results are consistent with a mechanism involving hydrogen atom abstraction from C-5 of the thiazolidinone ring, presumably mediated by oxygen, to afford a stabilized radical that can either react with oxygen to produce hydroperoxide **A** or combine to produce dimer **48**. The absolute requirement for an L-configured amino acid coupled with the high sensitivity of potency to changes in the amine capping element suggested that this region of the molecule made an important contribution to the antiviral activity observed with dimer **48**. This appreciation led to the hypothesis that excision of the iminothiazolidinone heterocycles would simplify the NS5A-inhibiting pharmacophore in **48** to that of the palindromic bibenzyl derivative **54**, as depicted in Figure 1. Synthetic accessibility facilitated the testing of this hypothesis, and **54**, a compound quite stable to replicon assay conditions, demonstrated potent inhibition, EC_{50} = 30 nM.[28] Furthermore, the more rigidly disposed stilbene **55** proved to be an even more potent inhibitor of the 1b HCV replicon with an $EC_{50} = 0.086 \pm 10$ nM ($n = 4$).[28,29] As is evident from Table 4, SAR for this new class of stilbene inhibitors closely resembled that of the iminothiazolidinones. Replacing the phenylacetic acid amine cap with Cbz (compound **57**) effected a 100-fold loss in potency, similar in magnitude to that observed between **18** and **2**, while the L-configuration was again essential for potency based on the comparison of **56** and **58** with the natural proline derivatives **55** and **57**. Additional profiling of stilbene **55** in the Y93H replicon revealed reduced potency, EC_{50} = 4 μM, confirming its association of inhibition with the NS5A protein. In a genotype 1a replicon, stilbene **55** was found to be inactive, EC_{50} > 10 μM, providing a clear objective for the next phase of optimization.

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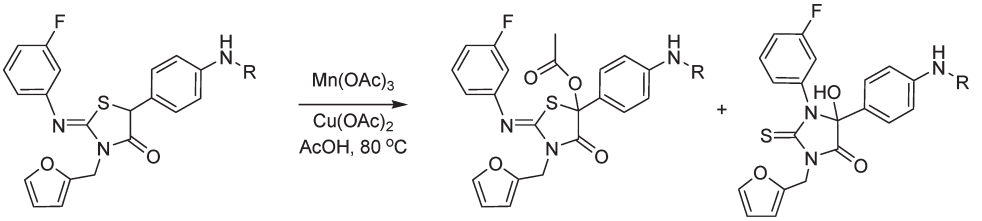
Scheme 3. Radical Generation, Oxidation, and Trapping with Acetic Acid

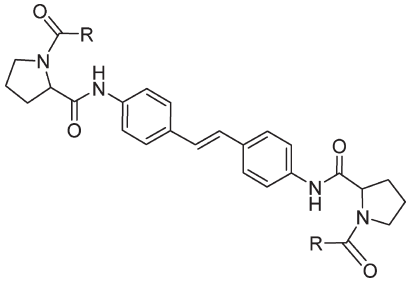


49 R = Ac
2 R = L-AlaCO$_2$CH$_2$Ph
18 R = L-AlaCOCH$_2$Ph

50 R = Ac
52 R = L-AlaCO$_2$CH$_2$Ph
53 R = L-AlaCOCH$_2$Ph

51 R = Ac

Table 4. SAR Associated with Stilbene-Based HCV NS5A Inhibitors



Cmpd	Proline moiety	R	EC$_{50}$ (μM)[a]
55		CH_2Ph	0.000086
56		CH_2Ph	>10
57		OCH_2Ph	0.010
58		OCH_2Ph	>10

[a] EC$_{50}$ values are averages of at least two independent determinations.

BMS-790052 (**1**) is a first-in-class inhibitor for HCV NS5A with high selectivity, picomolar potency, and broad genotype coverage *in vitro* that likely acts by disrupting a replication−protein assembly process critical to viral replication.[5] The synthesis of stilbene **55** was a critical step in the design of BMS-790052 (**1**), a compound that has established the clinical relevance of interfering with the function of NS5A as an approach to the treatment of HCV infection.[5] Interestingly, the discovery of symmetry within HCV NS5A inhibitors coincides well with X-ray crystallographic data subsequently published for the amino terminus of the NS5A protein.[30,31] In one report, NS5A residues 36−198 crystallized as a dimer forming a U-shaped structure with the inner surface lined with a preponderance of basic amino acids proposed to form a binding site for viral RNA.[30] The residues conferring resistance to inhibitor **55** are proximal to the amino terminus of NS5A and are located between the protein and the membrane, suggesting that the symmetrical compounds bind across the dimer interface.[5] Although symmetry is not essential for inhibition of HCV replication in genotype 1b replicon, as reported elsewhere, genotype 1a inhibition is most optimally achieved in symmetrically disposed compounds.[32]

In summary, we describe here fundamental aspects associated with the HCV inhibitory activity of a series of C-5-phenylated iminothiazolidinones discovered in a high throughput replicon screen, compounds that target the NS5A protein. The SAR observed for this chemotype was highly sensitive to the absolute configuration of the amino acid and to structural changes in the capping element at the amino terminus, and it was consistent between the alanine and proline series. Chemical reactivity at the C-5 position of iminothiazolidinones in DMSO was based on a propensity to form radicals at this site, resulting in oxidation *via* reaction with oxygen or the formation of dimeric species upon radical combination. The precise SAR associated with the amino acid moiety suggested that this element was a critical determinant of biological activity and lead to a hypothesis that the pharmacophore inherent in **48** could be simplified to that of a symmetrical bibenzyl derivative, an idea initially vindicated upon synthesis and evaluation of **54**. The potent inhibition of HCV replication in the replicon assay by symmetrically disposed stilbene **55** ultimately lead to the design of BMS-790052 (**1**), the compound that established proof-of-concept for HCV NS5A inhibition as a treatment of HCV infection.

■ ASSOCIATED CONTENT

Supporting Information. Experimental details for synthetic procedures and associated chemical data for compounds **2**−**58** and the experimental protocol used to evaluate inhibitors in the replicon assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) The diastereomers at the C-5 position of **2** were separated by chiral prep HPLC but observed to rapidly equilibrate due to facile keto−enol tautomerism (see Supporting Information for LC trace).

(14) Compounds **2**−**15** were prepared in library format using parallel synthesis methodology and subject to minimal purification. All compounds were of at least 70% purity, and MWs were confirmed by LCMS.

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(16) See Supporting Information for HPLC stability studies and experimental details. In DMSO, compounds **2** and **18** underwent complete conversion to **46** and **47**, respectively. All compounds were dissolved and diluted in DMSO prior to evaluation in replicon and preincubation in media.

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(24) Chemical shifts were consistent with the presence of an imine bond (not present in **51**), while H−N long-range correlation indicated an amide nitrogen (see Supporting Information).

(25) Dimers derived from **2** and **49** were detectable by LCMS in low amounts, <1%. Dimer isolated from media was obtained from 1 L scale incubation of **18** at a concentration of 100 μM and yielded 1.1 mg each of the two isomers (see ref 17 for a more detailed account). The synthetic material was identical with the more mobile isomer and demonstrated an EC_{50} = 2 nM. Dimer and acetates **52** and **53** were formed as single compounds with no evidence of diastereomers being formed (see Supporting Information).

(26) For the radical dimerization process, see: Obata, N.; Niimura, K. Oxidative Dehydrodimerization of *N*-Acyl α-Amino-acids: Synthesis of Novel Di-αα′-amino-acid Derivatives. *Chem. Commun.* **1977**, 238–239.

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