N-Substituted 3-Acetyltetramic Acid Derivatives as Antibacterial Agents

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In order to expand the structure–activity relationship of tetramic acid molecules with structural similarity to the antibiotic reutericyclin, 22 compounds were synthesized and tested against a panel of clinically relevant bacteria. Key structural changes on the tetramic acid core affected antibacterial activity. Various compounds in the *N*-alkyl 3-acetyltetramic acid series exhibited good activity against Gram-positive bacterial pathogens including *Bacillus anthracis*, *Propionibacterium acnes*, *Enterococcus faecalis*, and both Methicillin-sensitive and -resistant *Staphylococcus aureus*.

Introduction

The ability to effectively treat bacterial infectious diseases through antimicrobial chemotherapy is being severely undermined by the widespread emergence of antibiotic resistance among bacterial pathogens. These drug-resistant bacteria are a major cause of morbidity and mortality in both hospital and community settings.[1] Thus, there is an urgent need to develop new chemotherapeutic agents to treat increasingly common, clinically relevant, drug-resistant strains such as *Staphylococcus aureus*, *Streptococcus pyogenes, Streptococcus pneumoniae, Mycobacterium tuberculosis*, and *Enterococcus faecalis*.[2] One approach to develop novel therapeutic agents is by employing diversity-oriented synthesis of natural product-like libraries.[3] Naturally occurring tetramic acid derivatives are of great interest because of their broad spectrum of antibacterial activity.[4] However, there is currently no drug in the market that contains this type of scaffold.[4] Tetramic acid is a key constituent of various natural products such as reutericyclin **1**,[5] tenuazonic acid **2**,[6] streptolydigin **3**,[7] "**4** (PF1052)",[8] and erythroskyrine **5**[9] (Figure 1), all of which exhibit antibacterial activity. In most cases, the tetramic acid core is present as a 3-acyl derivative. Although quite a number of studies have focused on synthesizing and isolating naturally occurring tetramic acids, there are only a few reports[6,10–12] regarding the structure–activity relationships (SAR) of these compounds with emphasis on their antibacterial properties. Reutericyclin was isolated from *Lactobacillus reuteri*,[13] and the chemical synthesis of this molecule has been demonstrated using racemic[14] and enantiomerically specific routes.[15] However, it has an N-substituted unstable α,β-unsaturated acyl side chain attached to the teramic acid core. In the present study, the SAR of N-substituted tetramic acids, similar in structure to reutericyclin, was explored by introducing a variety of chemically stable *N*-alkyl, *N*-aryl or *N*-alkenyl side chains and evaluating their antibacterial properties. A series of N-substituted 3-cyanotetramic acids (**6**) and N-substituted 3-acetyltetramic acids (**7**) (Figure 2) were successfully synthesized using a solid-supported reagent. The antibacterial susceptibility of these compounds and the structure–activity relationships developed are discussed herein.

Chemistry

To investigate the importance of acyl substituents at the 3-position on the tetramic acid core, N-substituted 3-cyanotetramic acid derivatives **9a**−**g** (Scheme 1) were synthesized using the method described by Kulkarni et al.[16] L-Amino acid ester salts were used as starting materials for the synthesis of compounds **9a**−**e** and D-amino acid ester salts were used as starting materials for the synthesis of compounds **9f**,**g**. N-Substituted secondary amines **8a**−**g** were synthesized by reductive amination reactions of the amino acid ester salts with various alkyl and aryl aldehydes in THF in the presence of $MgSO_4$ and Et_3N for 5 h, and the resulting imines were reduced using $NaBH_4$ in methanol for 30 min.[17] Subsequently, secondary amines **8a**−**g** were converted into their respective cyanoamides by reacting them with the cyanoacetic acid in DCM in the presence of DIC or DCC and HOBt at rt for 6 h to afford cyanoamide intermediates. The cyanoamides were then cyclized into tetramic acids by using Amberlyst A-26 hydroxide resin in methanol at rt for 2 h.[16] The use of a solid-phase resin during the cyclization step eliminates the need for column purification by acting both as a reaction catalyst and as scavenger resin for the product. The unreacted amides and other impurities were simply removed by subsequent washings with methanol. The final products were obtained by elution of the resin with methanol/ TFA at rt for 20 min.

To explore the importance of the substituents at the 1-position on reutericyclin, several stable N-substituted 3-acetyl tetramic acids were synthesized (Scheme 2). Secondary amines **8a**−**o** that were obtained by reductive amination were subsequently reacted with diketene in DCM in the presence of catalytic amounts of Et_3N at reflux temperature to yield their respective β-keto amides.[18] These amides were subsequently cyclized into 3-acetyltetramic acids[19] **10a**−**o** using Amberlyst A-26 hydroxide resin.[16] The structural determination of 3-acyltetramic acids is complex because they exist in different tautomeric forms. These tautomeric forms can exist in both internal and external pairs (Figure 3).[20] Internal tautomerization involves the rapid transfer of a hydroxyl proton, which is difficult to detect in an NMR time scale. However, NMR does detect the ratios of external tautomers. Steyn et al. have analyzed the X-ray crystallography data and reported that tetramic acids primarily exist as exoenol tautomer (**11d**) (Figure 3).[21] Thus, for the sake of consistency in the structures through out this paper, 3-acetyltetramic acids are represented as exoenol tautomer (**11d**).

Results and Discussion

To explore the structure–activity relationship of tetramic acids, 22 compounds were synthesized and evaluated for their antibacterial properties. The compounds were tested against a wide panel of clinically relevant bacteria consisting of *M. tuberculosis*, *Escherichia coli*, *S. aureus*, *E. faecalis*, *Bacillus anthracis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *S. pyo-*

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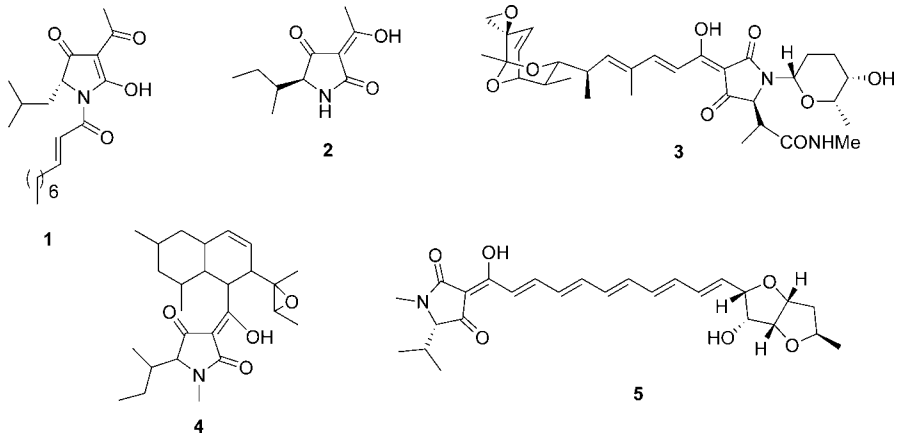


Figure 1. Structures of **1**, reutericyclin; **2**, tenuazonic acid; **3**, streptolydigin; **4**, PF1052; and **5**, erythroskyrine.

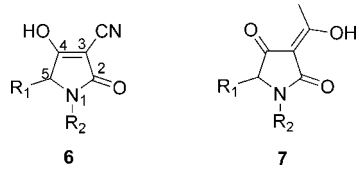


Figure 2. General structures of **6**, N-substituted 3-cyanotetramic acids, and **7**, N-substituted 3-acetyltetramic acids.

genes, *S. pneumoniae*, and *Propionibacterium acnes*. Results arising from the antibacterial screens reveal a clear SAR as it relates to the ability of different chemical structures to inhibit bacterial growth. None of the tetramic acids synthesized were active against *E. coli* and *P. aeruginosa* (MIC > 200 μg/mL), indicating that these compounds are primarily effective against Gram-positive bacteria. This finding is not surprising and may in part reflect the inability of these compounds to penetrate the complex outer membrane barrier of Gram-negative bacteria.[22] Compounds in the N-substituted 3-cyano series (**9a**−**g**) were inactive against all strains except for **9b**, which interestingly was the most active compound in the entire series of 22 compounds when tested against *M. tuberculosis* (MIC of 3.12 μg/mL).

The N-substituted 3-acetyltetramic acids (**10a**−**o**), in contrast to their cyano counterparts, were active against many clinically relevant Gram-positive bacterial species (Table 1), suggesting the requirement of an acidic proton for anti-Gram-positive activity. In this class of compounds, N-substitution appears to play a critical role in engendering antibacterial activity. Tetramic acids bearing *n*-decyl side chains were generally the most active species (**10b**,**i**,**n**,**o**). Nevertheless, compounds possessing other lipophilic substituents such as the bicyclic ring system (**10l**) and the branched unsaturated systems (**10m**) also demonstrated good antibacterial activities. Even replacement of *n*-decyl with a biphenyl ring system (**10k**) did not affect antibacterial activity; however, reduced activity was associated with compound **10a**, which carries a single phenyl ring and the compounds with aryl ring systems (**10d** and **10e**). Shortening of chain lengths from *n*-decyl (**10b**) to *n*-butyl (**10j**) also led to decreased activity in all strains except *E. faecalis* and *P. acnes*. However, this substitution did seem to lower the toxicity of the compound (IC_{50} 254 μg/mL). It appears that R_1 substitutions at the 5-position do not play a major role in determining antibacterial activity since the isobutyl (**10b**), isopropyl (**10i**), and benzyl (**10n**) side chain tetramic acids exhibited similar trends in their activities. The long *n*-decyl chain most likely facilitates compound entry into the cell membrane where the compounds are believed to act.[23,24] *S. pyogenes* and *S. pneumoniae* were the least susceptible of the classical Gram-positive species and displayed a trend very similar to *M. tuberculosis*. Both streptococci and *M. tuberculosis* possess complex cell wall envelopes that might have enabled these to be more refractory to penetration by tetramic acids that the other tested Gram-positive species.[25,26] We also observed that stereochemistry did not affect the antibacterial properties of tetramic acid compounds. In the N-substituted 3-cyanotetramic acids series, the compounds that were synthesized from D-amino acids ester salts (**9f** and **9g**) and their corresponding l-amino acid ester salt analogues (**9e** and **9c**) did not exhibit any antibacterial properties. Similarly, in the 3-acetyltetramic acids, the compounds synthesized from D-amino acids ester salts (**10f**,**g**,**o**) and their corresponding L-amino acid ester salt analogues (**10e**,**c**,**b**) had similar activities.

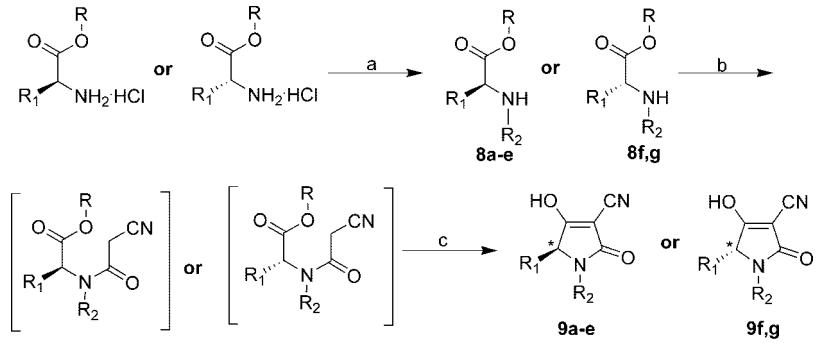
The selectivity indices for the compounds were calculated as the ratio of the cytotoxic IC_{50} against Vero monkey kidney cell line and the MIC against the wild type drug-sensitive strain *S. aureus* 8325. Compounds demonstrating good antimicrobial action (**10b**,**i**−**n**,**o**) also exhibited a favorable selectivity index ($\geq$10), with the exception of **10i** (SI = 8.8). These findings indicate that further studies are warranted to investigate their usefulness as antibacterials agents in the treatment and prevention of infections caused by Gram-positive pathogens.[27]

Conclusions

In summary, we have developed tetramic acid molecules which exhibit good antibacterial properties. Compounds in this series were easily synthesized enabling facile development of this class. The synthesis of tetramic acids bearing a cyano group at the third position has previously been reported.[16] However, we have shown for the first time that incorporation of the 3-cyano group hinders the antibacterial activity of tetramic acid compounds. This study found that the 3-acetyl functionality is important for the antibacterial activity of tetramic acids and defines the 3-acetyltetramic acid derivatives as an interesting class of compounds with good activity against Gram-positive pathogens.

The antimicrobial mechanism of action for tetramic acids for the most part is not well characterized. A prior study with reutericyclin has shown that its antibacterial action results from the molecule acting as a proton ionophore to cause dissipation of the transmembrane pH of bacteria.[23,24] The 3-acetyl derivatives described in this study are closely related to reutericyclin but possess chemically stable *N*-alkyl, *N*-aryl, or *N*-alkenyl side chains. It is therefore plausible that the 3-acetyl group of compounds, in contrast with the 3-cyano derivatives, enables the translocation of protons across the membranes of susceptible bacteria, thereby

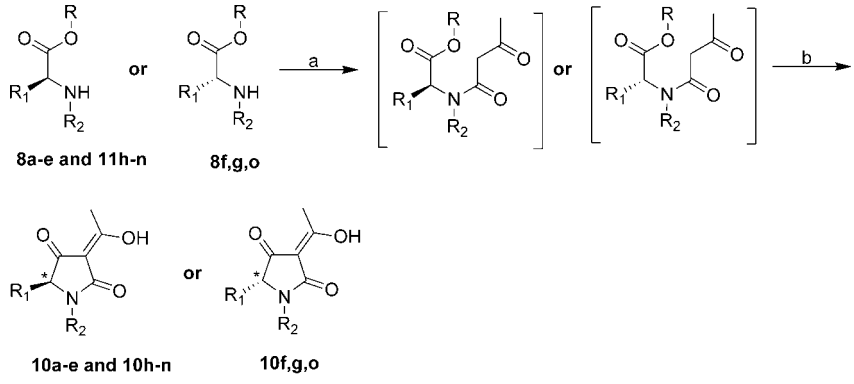
Scheme 1[a]



R = CH_3 or C_2H_5;
a R_1 = *sec*-butyl; **b, d, e, f** R_1 = *iso*-butyl; **c, g** R_1 = benzyl;
a, c, g R_2 = benzyl; **b** R_2 = *n*-decyl; **d** R_2 = *m*-methoxy benzyl; **e, f** R_2 = *p*-fluoro benzyl.

[a] Reagents: (a) (i) aldehydes, Et_3N, $MgSO_4$, THF, rt, 5 h; (ii) $NaBH_4$, MeOH, rt, 30 min; (b) cyanoacetic acid, HOBt, DIC or DCC, CH_2Cl_2, rt, 6 h; (c) (i) Amberlyst A-26 hydroxide resin, MeOH, rt, 2 h; (ii) MeOH, TFA, rt, 20 min. *Indicates racemizations might have occurred during synthesis.

Scheme 2[a]



R = CH_3 or C_2H_5;
a R_1 = *sec*-butyl; **b, d, e, f, j, k, l, m, o** R_1 = *iso*-butyl; **c, n, g, h** R_1 = benzyl; **i** R_1 = *iso*-propyl;
a, c, g R_2 = benzyl; **b, i, n, o** R_2 = n-decyl; **d** R_2 = *m*-methoxy benzyl; **e, f** R_2 = *p*-fluoro benzyl; **h** R_2 = ethyl; **j** R_2 = *n*-butyl; **k** R_2 = *p*-phenyl benzyl; **l** R_2 = myrtenyl; **m** R_2 = citronellyl.

[a] Reagents: (a) diketene or 50% diketene in CH_2Cl_2, CH_2Cl_2, Et_3N, 6 h, reflux; (b) (i) Amberlyst A-26 hydroxide resin, MeOH, rt, 2 h; (ii) MeOH, TFA, rt, 20min. *Indicates racemizations might have occurred during synthesis.

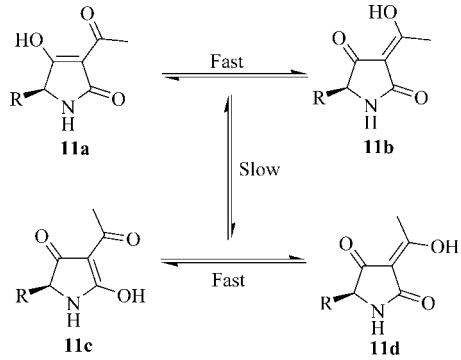


Figure 3. Structures of internal and external pairs of tetramic acids.

altering pH dependent intracellular reactions. The chemical structure of side chains introduced at the N-substituted position also appears to influence the antibacterial action of 3-acetyl tetramic acids. Activity was improved by introducing more lipophilic substituents, a property that may reflect better partitioning of bacterial cell membranes by these molecules and is consistent with the proposed antibacterial mode of action of this type of tetramic acids as proton ionophores.

Previously, it has been suggested that the challenge in the development of tetramic acids as antibiotic agents lies in the potential toxicity of these compounds.[4] Results arising from this study, however, showed that the cytopathic effects of this class can be modulated by introducing different groups at the N-substituted position. Additional studies are underway to characterize the mode of action of these compounds and investigate their potential for medical use as topical agents for the control of superficial infections caused by Gram-positive pathogens.

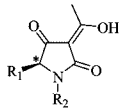
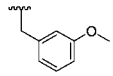
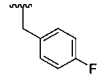
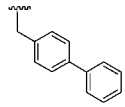
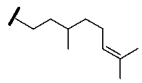
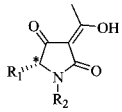
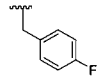
Experimental Section

General Procedure for Preparation of Secondary Amines (8a−o). To a stirred solution of amino acid HCl salt (1 equiv) in THF were added $MgSO_4$ (1.7 equiv), aldehyde (2 equiv), and Et_3N (1 equiv). The reaction was then allowed to stir at rt under argon for 5 h. The reaction mixture was then filtered and the eluent evaporated to give the crude imine intermediate. The imine was directly redisolved in methanol, and sodium borohydride (2 equiv) was slowly added to the reaction mixture. The reaction was stirred at rt for 30 min before being quenched with excess 1 N NaOH and extracted with ethyl acetate. The ethyl acetate extracts were combined, washed with brine, dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by flash column chromatography using a petroleum ether to ethyl acetate gradient elution to afford pure products.

The preparation of (2*S*,3*S*)-methyl 2-(benzylamino)-3-methylpentanoate (**8a**) is given as a representative example.

(2*S*,3*S*)-Methyl 2-(Benzylamino)-3-methylpentanoate (8a). Synthesized according to the above general procedure using L-isoleucine methyl ester hydrochloride (1 g, 5.5 mmol), THF (20 mL), $MgSO_4$ (1.12 g, 9.35 mmol), benzaldehyde (1.12 mL, 11.0 mmol), Et_3N (767 μL, 5.5 mmol), sodium borohydride (416 mg, 11.0 mmol),

Table 1. Structures and Activities of N-Substituted 3-Acetyltetramic Acid Derivatives[a]

General Structure	No.	R_1	R_2	Activity µg/mL										Selectivity index
				TB	BA	BS	EF	MRSA	MSSA	PA	SP	SPn	Toxicity IC_{50}	
 (10a-e and 10h-n)	**10a**			50	3.12	25	6.25	6.25	6.25	6.25	>200	50	26.3	4.2
	10b			12.5	0.8	0.8	1.6	0.8	0.4	0.8	50	6.25	14.7	36.8
	10c			>200	25	25	100	25	12.5	12.5	>200	100	75.7	6.1
	10d			100	25	25	100	25	12.5	12.5	200	50	40.0	3.2
	10e			100	12.5	25	50	25	12.5	6.25	100	50	27.8	2.2
	10h			>200	50	>200	>200	>200	200	100	>200	>200	NA	NA
	10i			12.5	0.8	0.4	0.8	0.8	0.8	0.4	25	6.25	7.0	8.8
	10j			100	12.5	6.25	1.6	6.25	3.12	0.8	>200	100	254.0	81.4
	10k			50	0.4	0.4	0.8	0.8	0.2	0.2	25	12.5	19.7	98.5
	10l			100	0.8	1.6	1.6	1.6	1.6	0.8	25	50	35.4	22.1
	10m			200	0.4	0.8	0.8	0.8	0.4	0.4	25	3.25	7.3	18.3
	10n			50	0.2	0.4	0.8	0.8	0.2	0.4	50	12.5	21.1	105.5
 (10f,g,o)	**10f**			100	12.5	12.5	50	12.5	12.5	12.5	100	25	35.3	2.8
	10g			>200	25	25	100	25	25	25	200	50	80.6	3.2
	10o			25	0.8	0.8	1.6	0.8	0.4	0.8	50	6.25	6.3	15.8

[a] Key: TB, *M. tuberculosis* H37Rv; BA, *B. anthracis* Sterne 34F2; BS, *B. subtilis* ATCC 23857; EF, *E. faecalis* ATCC 33186; MRSA, methicillin-resistant *S. aureus* ATCC 33591; MSSA, methicillin sensitive *S. aureus* 8325 ATCC 35556; *P. acnes* ATCC 6919; SP, *S. pyogenes* ATCC 700294; SPn, *S. pneumoniae* DAW30; cytotoxicity IC_{50}, concentration which reduces viability of Vero kidney cells by 50%; selectivity index (SI), cytotoxicity IC_{50} divided by MIC against the methicillin sensitive strain *S. aureus* 8325; NA, not assessed.

and methanol (30 mL) to give **8a** (880 mg, 68%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88–0.943 (6H, m), 1.16–1.3 (1H, m), 1.55–1.64 (1H, m), 1.68–1.88 (2H, m), 3.13 (1H, d, J = 6.10 Hz), 3.62 (1H, d, J = 12.93 Hz), 3.74 (3H, s), 3.84 (1H, d, J = 12.93 Hz), 7.24–7.29 (1H, m), 7.31–7.39 (4H, m). ESI-MS: 258.0 (M + 23).

General Procedure for Preparation of N-Substituted 3-Cyanotetramic Acid Derivatives (9a−g). To a solution of substituted amino acids (1 equiv) in CH_2Cl_2 were added cyanoacetic acid (1.12 equiv), HOBt (1.12 equiv), and either DIC or DCC (1.4 equiv), and the mixture was stirred for 6 h at rt. The reaction mixture was subsequently filtered, diluted with CH_2Cl_2, and washed with water, saturated $NaHCO_3$, and brine. The organic fraction was dried over Na_2SO_4 and concentrated. This was purified by flash column chromatography using a petroleum ether to ethyl acetate gradient elution to afford amides. To the solution of amide (1 equiv) in methanol (10 mL) was added Amberlyst A-26 resin (4.2 meq/gm, 3 equiv), and the reaction was stirred at rt under argon for 2 h. Resin containing the product was filtered and washed with methanol (3 × 10 mL). The resin was then stirred for 30 min with methanol (10 mL) and

TFA (400 μL), filtered, and washed with methanol (3 × 10 mL). Concentration of the eluent afforded the desired products.

The preparation of (*S*)-1-benzyl-5-*sec*-butyl-4-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (**9a**) is given as a representative example.

(*S*)-1-Benzyl-5-*sec*-butyl-4-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (9a). Compound **9a** was synthesized according to the above general procedure using (2*S*,3*S*)-methyl 2-(benzylamino)-3-methylpentanoate **8a** (880 mg, 3.74 mmol), CH_2Cl_2 (20 mL), cyanoacetic acid (358 mg, 4.19 mmol), HOBt (566 mg, 4.19 mmol), and DCC (1.08 g, 5.23 mmol) to give amide (650 mg, 57%). To the solution of amide (180 mg, 0.596 mmol) in methanol (10 mL) was added Amberlyst A-26 resin (425 mg, 1.78 mmol), and the reaction was carried out as described in the above general procedure to give **9a** (140 mg, 87%). ^{1}H NMR (500 MHz, CD_3OD): δ 0.78 (3H, d, J = 6.86 Hz), 0.88 (3H, d, J = 7.96 Hz), 1.42–1.6 (2H, m), 1.9–2.08 (1H, m), 3.94 (1H, d, J = 3.02 Hz), 4.18 (1H, d, J = 15.37 Hz), 5.01 (1H, d, J = 15.37 Hz), 7.27 (2H, d, J = 6.86 Hz), 7.72–7.33 (1H, m), 7.34–7.39 (2H, m). ESI-MS: 268.9 (M − 1). IR ν_{max}(cm^{-1}): 2225.86, 1642.35, 1570.15 cm^{-1}. $[\alpha]^{26.2}_D$ −62.2 (c = 1, MeOH). HPLC1: t_R 5.87 min, purity >99%. HPLC2: t_R 5.11 min, purity 98%.

General Procedure for Preparation of N-Substituted 3-Acetyltetramic Acids (10a−o). To a solution of substituted amino acid (1 equiv) in CH_2Cl_2 were added diketene (1 equiv) and Et_3N (five drops), and the mixture was then heated under reflux for 6 h. The reaction mixture was then cooled, diluted with CH_2Cl_2, and washed with dilute hydrochloric acid followed by water. The CH_2Cl_2 fraction was dried over Na_2SO_4 and concentrated. This was then purified by flash column chromatography using a petroleum ether to ethyl acetate gradient elution to afford the desired intermediate products that were then used directly in the next step. To the solution of amide (1 equiv) in methanol (10 mL) was added Amberlyst A-26 resin (4.2 mequiv/g, 3 equiv), and the reaction was stirred at rt under argon for 2 h. The product containing resin was filtered and washed with methanol (3 × 10 mL). The resin was then stirred for 30 min with methanol (10 mL) and TFA (400 μL), filtered, and washed with methanol (3 × 10 mL). Concentration of the eluent afforded the desired products.

The preparation of (*S*,*Z*)-1-benzyl-5-*sec*-butyl-3-(1-hydroxyethylidene)pyrrolidine-2,4-dione (**10a**) is given as a representative example.

(*S*,*Z*)-1-Benzyl-5-*sec*-butyl-3-(1-hydroxyethylidene)pyrrolidine-2,4-dione (10a). Compound **10a** was synthesized according to the above general procedure using (2*S*,3*S*)-methyl 2-(benzylamino)-3-methylpentanoate **8a** (500 mg, 2.12 mmol), CH_2Cl_2 (30 mL), diketene (165 μL, 2.12 mmol), and Et_3N (five drops) to give amide (420 mg, 62%). To the solution of amide (420 mg, 1.31 mmol) in methanol (10 mL) was added Amberlyst A-26 resin (936 mg, 3.93 mmol), and the reaction was carried out as described in the above general procedure to give **10a** (310 mg, 82%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.78–0.94 (6H, m), 1.5–1.66 (2H, m), 1.9–2.2 (1H, m), 2.46 (2.2H, s, Me 3-acetyl major tautomer), 2.58 (0.8H, s, Me 3-acetyl minor tautomer), 3.59 and 3.76 (1H, 2ds, J = 3.29 Hz), 3.94–4.4 (1H, m), 5.24–5.38 (1H, m), 7.22–7.28 (2H, m), 7.31–7.42 (3H, m). ^{1}H NMR (500 MHz, CD_3OD): δ 0.76 (3H, d, J = 6.86 Hz), 0.87 (3H, t, J = 7.41 Hz), 1.46–1.66 (2H, m), 1.9–2.4 (1H, m), 2.47 (3H, s), 3.72–3.8 (1H, bs), 4.21 (0.81 H, d, J = 15.1 Hz) and 4.35 (0.19 H, d, J = 15.31 Hz), 5.01 (0.81H, d, J = 15.37 Hz), 5.12 (0.19 H, d, J = 15.1 Hz), 7.29–7.41 (5H, m). ESI-MS: 286 (M − 1). IR ν_{max} (cm^{-1}): 1709.26, 1615.06 cm^{-1}. $[\alpha]^{27.3}_D$ −87.0 (c = 1%, MeOH). HPLC1: t_R 7.17 min, purity >99%. HPLC2: t_R 6.10 min, purity 97%.

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Supporting Information Available: Experimental procedure for the synthesis and spectral data of compounds **8b−o**, **9b−g**, and **10b−o** and experimental procedures for MIC determination and cytotoxicity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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