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Novel Natural Products from Soil DNA Libraries in a Streptomycete Host

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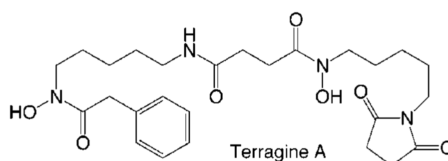
ABSTRACT



As a route to accessing the potential chemical diversity of uncultivable microbes from the soil, combinatorial biosynthetic libraries were constructed by cloning large fragments of DNA isolated from soil into a *Streptomyces lividans* host. Four novel compounds, terragines A (1), B (2), C (3), and D (4), were isolated from recombinant 436-s4-5b1, and another novel compound, terragine E (5), was isolated from 446-s3-102g1. The structures were determined by a combination of spectroscopic techniques, primarily 2D NMR.

The production of antibiotics and other pharmaceuticals by bacteria and fungi is constrained because the majority of the microbial species in the biosphere cannot be grown in the laboratory. It has been estimated that only 0.1−1.0% of all microbes can be studied by conventional microbial methods.[1] A genetic approach to gaining access to uncultivable microbes has been developed in which total DNA is isolated from an environmental sample, ligated into a vector, and introduced into a surrogate host for expression.[2] The isolation methods, vectors, and host are chosen such that DNA fragments large enough to contain partial or complete biosynthetic pathways are cloned. Each environmental sample provides a unique collection of DNA sequences, which are expected to encode extensive molecular diversity. Thus, large numbers of recombinant microorganisms are generated and screened for the production of novel molecules by employing chemical and biological methods of analysis. We now report on the isolation and structural determination of novel compounds from some *Streptomyces lividans* recombinants.

A rapid HPLC-ESIMS screening method and downstream database treatment were developed in order to identify transformants that might contain novel compounds. In the first stage, 1020 recombinants were screened. After evaluation of the HPLCMS data, recombinants 436-s4-5b1 and 446-s3-102g1 were found to produce potentially novel compounds with ESIMS ions at *m/z* 585, 519, 479, 319, and 279.

The biotransformant 436-s4-5b1 was fermented on solid ATCC media for 10 days at 30 °C.[3] Ethyl acetate extraction of the resulting combined cells and medium gave a crude extract (5.0 g) that was partitioned between hexane and 90% aqueous methanol. Evaporation of the aqueous methanol portion in vacuo gave 4.18 g of a crude oil that was fractionated by Sephadex LH20 size exclusion chromatography (eluent: MeOH). Fractions 3−7 (2.34 g) from the LH20 chromatography, containing compounds with the

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(1) Goksoyr, T. V. J.; Daae, F. L. *Appl. Environ. Microbiol*. **1990**, *56*, 782−787.
(2) Yap, W. H.; Li, X.; Soong, T. W.; Davies, J. E. J. *Industrial Microbiol*. **1996**, *17*, 179−184

(3) *Streptomyces lividans* 436-s4-5b1 has been deposited in the ATCC (Accession number 700989).

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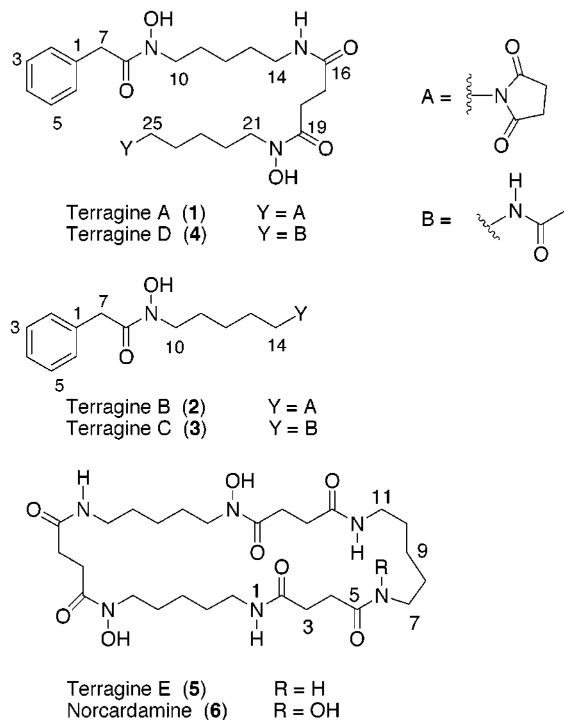
targeted molecular weights, were subsequently subjected to repeated reversed-phase flash chromatography (eluent: MeOH/H_2O step gradient) and reversed-phase HPLC to obtain terragine A (**1**) (MH^+ *m/z* 519, 4.1 mg), terragine B (**2**) (MH^+ *m/z* 319, 1.6 mg), terragine C (**3**) (MH^+ *m/z* 279, 2.0 mg), terragine D (**4**) (MH^+ *m/z* 479, 0.5 mg), and nocardamine (**6**) (6.2 mg). Terragine E (**5**) (MH^+ *m/z* 585, 3.0 mg) was isolated from cultures of another recombinant, 446-s3-102g1, using the same protocol.



Nocardamine (**6**), which has been previously isolated from *Nocardia* sp., *Pseudomonas stutzeri*, and *Streptomyces hygroscopicus gedanus*,[4] was readily identified by examination of its NMR[5] and ESIMS data.

Terragine A (**1**) gave $[M + H]^+$ and $[M + Na]^+$ ions in the HRFABMS at *m/z* 519.2798 (ΔM −4.0 mmu) and 541.2612 (ΔM −4.8 mmu), respectively, that were consistent with a molecular formula of $C_{26}H_{38}N_4O_7$, requiring 10 sites of unsaturation. The ^{13}C NMR/APT/HMQC data obtained for **1** contained only 22 resolved ^{13}C resonances (6 × C, 2 × CH, 14 × CH_2) indicating the presence of symmetry in the molecule.[6] Four of the quaternary carbon resonances had chemical shifts (δ 170.5, 171.2, 171.9, 177.7) suggesting they could be assigned to carboxylic acid or carboxylic acid derivative (ester or amide) carbonyls. A monosubstituted phenyl ring, which accounted for a portion of the symmetry in terragine A (**1**), was readily identified by the observation of ^{13}C NMR resonances at δ 136.0 (C-1), 126.1 (C-4), 129.4 (C-2/C-6), and 128.0 (C-3/C-5) that showed the expected HMQC and HMBC correlations to ^1H NMR resonances at δ 7.27 (H-3/H-5, t, $J = 7.3$ Hz) and 7.21 (H-2/H-4/H-6, m). Additional HMBC correlations observed between a two-proton resonance at δ 3.68 (s, H-7) and carbon resonances at δ 170.5 (C-8), 136.0 (C-1), and 129.4 (C-2/C-6) demonstrated that the phenyl ring was part of a phenylacetyl residue.

$^1H-^1H$ COSY correlations revealed two linear spin systems in **1** containing the H-10/H-11/H-12/H-13/H-14/NH-15 and H-21/H-22/H-23/H-24/H-25 resonances, respectively. The chemical shifts of the ^1H resonances in the H-10/H-11/H-12/H-13/H-14/NH-15 spin system and the chemical shifts of the ^{13}C resonances to which they were correlated in the HMQC spectrum of terragine A (**1**) were virtually identical to the observed ^1H and ^{13}C chemical shifts for the C-7 to NH-12 fragment of norcardamine (**6**),[3] indicating the presence of identical mono-*N*-hydroxylated diaminopentane substructures in both molecules. Likewise, the chemical shifts of the protons and their attached carbons in the H-21/H-22/H-23/H-24/H-25 spin system of **1** were also nearly identical to the corresponding ^1H and ^{13}C chemical shifts in the C-7 to NH-12 fragment of norcardamine (**6**), suggesting the presence of a second pentanediamine fragment in terragine A. However, COSY data showed that neither of the nitrogen atoms (N-20, N-26) in the latter pentanediamine residue were protonated.

COSY correlations observed between a pair of methylene ^1H resonances at δ 2.24 (H-17) and 2.75 (H-18) and HMBC correlations observed between each of these methylene ^1H resonances and the two carbonyl resonances at δ 171.2 (C-16) and 171.9 (C-19) identified an unsymmetrical succinyl residue in terragine A (**1**). HMBC correlations observed between the H-21 resonance (δ 3.42) and the C-19 resonance (δ 171.9) and between the H-14 resonance (δ 2.97) and the C-16 resonance (δ 171.2) showed that the succinyl residue was linked to the two pentanediamine fragments via amide bonds as in norcardamine (**6**). An additional HMBC correlation observed between the H-10 (δ 3.47) and C-8 (δ 170.5) resonances showed that the phenylacetyl residue was linked to N-9 via an amide bond. The C-10 (δ 47.1) and C-21 (δ 46.9) chemical shifts in **1** were nearly identical to the C-7 chemical shift (δ 46.8) in norcardamine (**6**), consistent with N-9 and N-20 hydroxylation in **1**.

The remaining fragment of terragine A (**1**) must account for an elemental composition of $C_4H_4O_2$, ^{13}C NMR resonances at δ 177.7 (CO, C-27/C-30) and 28.0 (CH_2, C-28/C-29), an ^1H NMR resonance at δ 2.59 (4H, s, H-28/H-29), and three sites of unsaturation. All of these features could

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(5) Norcardamine (**6**): ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm) 171.4 (C-2), 29.9 (C-3), 27.4 (C-4), 171.9 (C-5), 46.8 (C-7), 25.8 (C-8), 23.1 (C-9), 28.5 (C-10), 38.3 (C-11); ^1H NMR (600 MHz, DMSO-d_6) δ (ppm) 7.8 (NH, br-t, 6.0), 2.26 (H-3, t, 6.6), 2.57 (H-4, t, 6.6), 3.45 (H-7, t, 7.0), 1.47 (H-8, p, 7.0), 1.20 (H-9, p, 7.0), 1.35 (H-10, p, 7.0), 2.98 (H-11, dt, 6.0 and 7.0), 9.6 (OH, s).

(6) Terragine A (**1**): ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm) 136.0 (C-1), 129.4 (C-2/C-6), 128.0 (C-3/C5), 126.1 (C-4), 38.4 (C-7), 170.5 (C-8), 47.1 (C-10), 26.0 (C-11), 23.4 (C-12), 28.8 (C-13), 38.5 (C-14), 171.2 (C-16), 29.9 (C-17), 27.5 (C-18), 171.9 (C-19), 46.9 (C-21), 25.9 (C-22), 23.3 (C-23), 26.8 (C-24), 37.7 (C-25), 177.7 (C-27/C-30), 28.0 (C-28/C-29); ^1H NMR (600 MHz, DMSO-d_6) δ (ppm) 7.21 (H-2/H-4/H-6), 7.27 (H-3/H-5, t, 7.3), 3.68 (H-7, s), 3.47 (H-10, t, 7.0), 1.48 (H-11, p, 7.0), 1.19 (H-12, p, 7.0), 1.34 (H-13, p, 7.0), 2.97 (H-14, dt, 7.0, 6.0), 2.24 (H-17, t, 7.0), 2.75 (H-18, t, 7.0), 3.42 (H-21, t, 7.0), 1.48 (H-22, p, 7.0), 1.16 (H-23, p, 7.0), 1.42 (H-24, p, 7.0), 3.29 (H-25, t, 7.0), 2.59 (H-28/H-29, s), 7.76 (NH, t, 6.0), 9.9 (OH, br-s), 9.7 (OH, br-s).

be accommodated by a succinimide substructure involving N-26. HMBC correlations observed between the C-27/C-30 carbonyl resonance (δ 177.7) and both of the H-28/H-29 (δ 2.59) and H-25 (δ 3.29) methylene proton resonances confirmed the presence of the succimide moiety. Therefore, the structure of terragine A is **1**.

Having the structure of terragine A (**1**) as a model simplified the structure elucidation of terragines B (**2**), C (**3**), and D (**4**). Terragine B (**2**) gave an $[M + H]^+$ peak at m/z = 319.1652 in the HRFABMS, indicating a molecular formula of $C_{17}H_{22}N_2O_4$ (ΔM −2.0 mmu). Comparison of its NMR data[7] with the NMR data for terragine A (**1**)[5] showed that they were closely related compounds. Detailed analysis of the NMR data for **2** revealed that it contained only phenylacetyl, mono-*N*-hydroxylated diaminopentane (H-10/H-11/H-12/H-13/H-14), and succinimide substructures. HMBC correlations confirmed that the three fragments were linked in a linear fashion as shown in **2**.

Terragine C (**3**) gave an $[M + H]^+$ peak at m/z = 279.1705 in the HRFABMS consistent with a molecular formula of $C_{15}H_{22}N_2O_3$ (ΔM −1.3 mmu). Comparison of the 1D and 2D NMR data obtained for terragine C (**3**) with the NMR data for terragine B (**2**)[6] indicated that the two molecules differed simply by the replacement of the succinimide residue in **2** with an acetamide (^{13}C δ 168.9 (C, C-16), 22.6 (CH_3, C-17); ^1H δ 1.75 (3H, s, H-17)) residue in **3**.[8]

The molecular formula of terragine D (**4**), $C_{24}H_{38}N_4O_6$, was indicated by the presence of an $[M + H]^+$ peak at m/z 479.2871 (ΔM 0.3 mmu) in the HRFABMS. Comparison of the ^1H NMR data[9] obtained for terragine D (**4**) with the data for terragines A (**1**)[5] and C (**3**)[7] revealed that terragine D differed from terragine A simply by the replacement of the succinimide residue in **1** with an acetamide residue (δ_H 7.74 (NH-26), 1.75 (3H, s, H-28)) in **4**. It was not possible to obtain reliable ^{13}C NMR data for terragine D (**4**) because of the small quantity of material that was available.

The final novel compound, terragine E (**5**), was isolated from transformant 446-s3-102g1. Terragine E (**5**) gave an $[M + H]^+$ peak at m/z 585.3610 in the HRFABMS, consistent with a molecular formula of $C_{27}H_{48}N_6O_8$ (ΔM −0.3 mmu) that differed from the molecular formula of norcardamine (**6**) simply by the loss of one oxygen atom. The ^1H NMR spectrum of **5** contained a resonance that could be assigned to five NHs (δ 7.73) and another that could be assigned to only two NOHs (δ 9.61), which indicated that one of the hydroxamic acid hydroxyls in nocardamine (**6**) was replaced by a hydrogen atom in terragine E (**5**).[10]

In the current study, more than 1000 independent *S. lividans* recombinants were fermented in duplicate and examined for their small molecule products. Several compounds were produced by only a small number of the recombinants. For example, only 18 recombinants produced members of the terragine/norcardamine families. These 18 recombinants could be divided into 6 groups depending on their patterns of metabolite production (Table 1). It is noteworthy that nocardamine was not produced by group 1, and group 6 produced nocardamine without detectable terragine production. Variation in production by the different recombinant clones clearly indicates that terragine production was determined by the nature of the DNA insert. The DNA inserts in these strains are currently under examination, and it is not yet known if the terragines are produced by infrequent modification of host pathways or by the introduction of new biosynthetic functions.

Table 1. Summary of Terragine Production in *Streptomyces lividans* Recombinants

group	recombinants	1	2	3	4	5	6
	436-s4-10f2	*	*	*	*		
1	436-s4-31c3	*	*	*	*		
	436-s4-5a12	*	*	*	*		
	436-s4-10g2	*	*	*			
	436-s4-5g11	*	*	*	*	*	*
2	436-s4-5g12	*	*	*	*	*	*
	436-s4-5h3	*	*	*	*	*	*
	446-s4-31c4	*	*	*	*		*
3	436-s4-5b1	*	*	*	*		*
	436-s4-5h12	*	*	*		*	*
	436-s4-10b9	*	*	*			*
4	436-s4-5g6	*	*	*			*
	436-s4-5h2	*	*	*			*
	436-s4-5d2	*	*				*
	436-s4-8c4	*	*				*
	436-s4-5c1	*					*
5	436-s4-5c6	*					*
	436-s4-5e11	*					*
	601-s8-2a5						*
6	601-s9-134a12						*
	601-s9-134f7						*

Terragines contain the diaminopentane and succinyl biosynthetic building blocks found in nocardamine, but in addition their structures incorporate succinimide and phen-

(7) Terragine B (**2**): ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm) 136.0 (C-1), 129.3 (C-2/C-6), 128.0 (C-3/C-5), 126.1 (C-4), 38.4 (C-7), 170.5 (C-8), 46.9 (C-10), 25.8 (C-11), 23.2 (C-12), 26.8 (C-13), 37.6 (C-14), 177.7 (C-16/C-19), 27.9 (C-17/C-18); ^1H NMR (600 MHz, DMSO-d_6) δ (ppm) 7.20 (H-2, 4, 6, m), 7.28 (H-3/H-5, t, 7.3), 3.68 (H-7, s), 3.46 (H-10, t, 7.0), 1.50 (H-11, p, 7.0), 1.16 (H-12, p, 7.0), 1.44 (H-13, p, 7.0), 3.29 (H-14, t, 7.0), 2.57 (H-17, 18, s). 9.80 (OH, br-s).

(8) Terragine C (**3**): ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm) 136.0 (C-1), 129.4 (C-2, 6), 128.1 (C-3/C-5), 126.2 (C-4), 38.5 (C-7), 170.5 (C-8), 47.1 (C-10), 26.0 (C-11), 23.5 (C-12), 28.8 (C-13), 38.4 (C-14), 168.9 (C-16), 22.6 (C-17); ^1H NMR (600 MHz, DMSO-d_6) δ (ppm) 7.21 (H-2, 4, 6, m), 7.27 (H-3/H-5, t, 7.3), 3.69 (H-7, s), 3.48 (H-10, t, 7.0), 1.50 (H-11, p, 7.0), 1.19 (H-12, p, 7.0), 1.35 (H-13, p, 7.0), 2.96 (H-14, dt, 7.0, 6.0), 1.75 (H-17, s), 7.77 (NH-15, t, 6.0), 9.84 (OH, br-s).

(9) Terragine D (**4**): ^1H NMR (600 MHz, DMSO-d_6) δ (ppm) 7.21 (H-2/H-4/H-6, m), 7.28 (H-3, 5, t, 7.3), 3.68 (H-7, s), 3.46 (H-10, t, 7.0), 1.50 (H-11, p, 7.0), 1.20 (H-12, p, 7.0), 1.36 (H-13, p, 7.0), 2.95 (H-14, dt, 7.0, 6.0), 2.25 (H-17, t, 7.0), 2.55 (H-18, t, 7.0), 3.42 (H-21, t, 7.0), 1.50 (H-22, p, 7.0), 1.20 (H-23, p, 7.0), 1.36 (H-24, p, 7.0), 2.95 (H-25, t, 7.0), 1.78 (H-28, s), 7.74 (NH-15, 26, t, 6.0), 9.78 (OH, br-s), 9.58 (OH, br-s).

(10) Terragine E (**5**): ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm) 171.3 (C-2/C-24), 31.0 (C-3/C-4), 171.4 (C-5/C-13), 38.2 (C-7/C-11/C-22/C-33), 28.5 (C-8/C-10/C-21/C-32), 23.4 (C-9), 29.9 (C-14/C-25), 27.5 (C-15/C-26), 172.0 (C-16/C-27), 46.8 (C-18/C-29), 25.8 (C-19/C-30), 23.1 (C-20/C-31); ^1H NMR (600 MHz, DMSO-d_6) δ (ppm) 2.26 (H-3/H-4, s), 2.98 (H-7/H-11/H-22/H-33, dt, 6.0 and 7.0), 1.34 (H-8/H-10/H-21/H-32, p, 7.0), 1.20 (H-9/H-20/H-31, p, 7.0), 2.26 (H-14/H-25, t, 7.0), 2.57 (H-15/H-26, t, 7.0), 3.45 (H-18/H-29, t, 7.0), 1.47 (H-19/H-30, p, 7.0), 7.73 (5-NH), 9.61 (2OH).

ylacetamide fragments not found in norcardamine or the related compound desferrioxame B. The isolation of a family of terragines from the recombinants provides some insight into their biosynthesis. L-Lysine decarboxylation to form 1,5-diaminopentane has been demonstrated to be the first step in the biosynthetic pathway for the production of nocardamine and the linear siderophore desferrioxame B.[11] It appears that the early steps in the biosynthesis of the terragines involve the phenylacetylation and *N*-hydroxylation of one NH_2 group of 1,5-diaminopentane. Acetylation of the second amino group would give terragine C (**3**), while succinylation of the second amino group followed by cyclization to generate a succinimide would give terragine B (**2**). If instead of cyclizing the succinyl moiety couples with a second 1,5-diaminopentane residue and the new amide N is hydroxylated, then acetylation of the free amino terminus would give terragine D (**4**). Alternately, terragine A (**1**) would be produced if the chain was terminated by succinylation followed by cyclization to a succinimide. Nocardamine (**6**) and terragine E (**5**) would be obtained if three 1,5-diaminopentane and three succinoyl residues were alternately coupled, N-oxidized, and finally cyclized. The novel phenylacetylation and succinimide modifications observed in the terragines are presumably catalyzed by enzymes that are encoded by the heterologous DNA fragments in the recombinants.

Nocardamine (**6**) is a microbial siderophore that is used as a detoxification agent in iron and aluminum overload due to its excellent metal-complexing capabilities.[12] It also shows antimicrobial activity against mycobacteria. The terragines showed no in vitro inhibition of *Escherichia coli* or *Bacillus subtilis*. Other assays for biological activity are in progress.

In conclusion, to the best of our knowledge the terragines represent the first examples of novel metabolites isolated from soil DNA recombinants. Their production demonstrates that cloning of microbial DNA fragments isolated from soil samples and their expression in surrogate *Streptomycete* hosts can extend the spectrum of potentially useful compounds made by the host strain. Further studies using different inserts and different hosts should expand the possibilities of this approach.

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