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Generation of New Epothilones by Genetic Engineering of a Polyketide Synthase in *Myxococcus xanthus*

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Abstract Epothilones, potent cytotoxic agents and potential anticancer drugs, are complex polyketides produced by a modular polyketide synthase (PKS). The epothilone PKS genes were introduced and expressed in *Myxococcus xanthus* and engineered to generate novel unnatural natural products which can be used as new scaffolds for chemical modification. Inactivation of the KR domain in module 6 of the *epo* PKS resulted in accumulation of 9-oxoepothilone D and its isomer 8-*epi*-9-oxoepothilone D as the major products. Modification of the KR domain in module 4 resulted in the production of the expected compound 12,13-dihydro-13-oxoepothilone C in trace amounts, and the unexpected compound 11,12-dehydro-12,13-dihydro-13-oxoepothilone D as the major product. The other expected compound, 12,13-dihydro-13-oxoepothilone D, was not detected. The unexpected 13-oxo derivative produced indicates that the ER domain of module 5 has substrate-specificity requirements and suggests a second enzymatic role for the domain.

Keywords polyketide synthase, *Myxobacteria*, genetic engineering, ketoreductase

Introduction

Epothilones are potent cytotoxic compounds that are naturally produced by the myxobacterium *Sorangium cellulosum*. The most important congeners are epothilones A (**1**) and B (**2**), the end products of the biosynthetic pathway, and epothilones C (**3**) and D (**4**), which can be biosynthetically converted to epothilones A and B, respectively, by a cytochrome P450 epoxidase encoded by the gene *epoK* (Fig. 1) [1, 2]. Trace amounts of 37 other epothilone-like structures have been identified in fermentation broths of the producing organism [3]. The attractive preclinical profile of some epothilones has made them promising lead compounds as anticancer agents and has spurred a strong interest in obtaining derivatives to fully evaluate their therapeutic potential [4~6]. Epothilones D, B and B-derivatives are currently undergoing clinical trials. The epothilone polyketide synthase genes responsible for the biosynthesis of epothilones C and D have been cloned, sequenced, and moved into *Myxococcus xanthus*, a myxobacterium which is more amenable to genetic manipulation than the original host, and production of epothilone D at titers of greater than 25 mg/liter in *M. xanthus* using a fed-batch process has been achieved [2, 7, 8].

The backbone of all epothilones is produced from a type 1 modular polyketide synthase (PKS)-peptide synthetase. PKSs encode the synthesis of many important pharmaceuticals and have recently been the target of various molecular engineering approaches aimed at producing novel analogues or combinatorial libraries of novel polyketides [9, 10]. One approach to altering

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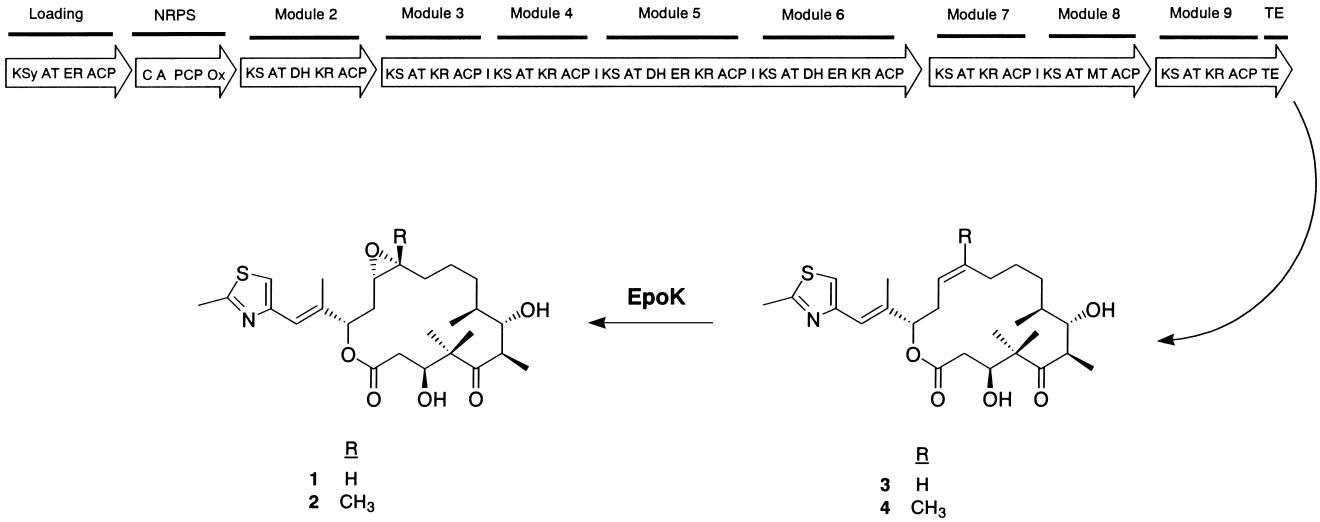

Fig. 1 Modular and domain organization of the epothilone polyketide synthase (PKS). The arrow indicates that compounds **3** or **4** are released from the PKS through the action of the TE domain. Abbreviations: KS^{Y}, β-ketoacyl ACP synthase containing a tyrosine substitution of the active-site cysteine; Ox, oxidase; KS, β-ketoacyl ACP synthase, AT, acyltransferase; ER, enoylreductase; ACP, acyl carrier protein; C, condensation; A, adenylation; PCP, peptidyl carrier protein; DH, dehydratase; KR, ketoreductase; MT, methyltransferase; TE, thioesterase.

polyketide structure is through inactivation of an existing domain such as an enoylreductase (ER), dehydratase (DH) or ketoreductase (KR) so that the corresponding step in the synthesis of the nascent polyketide chain is bypassed, resulting in the production of a compound with predicted structure. Homology modeling and mutational studies have led to the identification of amino acid residues that participate in catalysis in the KR, ER and DH domains, and to the understanding of the role of the KR domain in determining the stereochemistry of the side chains in the corresponding polyketide. The proposed residues involved in the catalytic function of the DH and ER domains in modular PKS have been examined in the epothilone PKS by replacing the corresponding residues in the DH or ER domains in module 5 [11, 12]. Here, we report the effect of replacing the putative corresponding residues in the KR domains of two *epo* PKS modules with those predicted to eliminate KR activity to produce novel epothilone analogues.

Results and Discussion

Modification in the KR4 Domain of the *epo* PKS

From homology analysis, it was predicted that replacement of the conserved Gly residue within a predicted Rossmann fold motif involved in cofactor binding and the conserved Tyr residue located in the active site would impair or eliminate KR activity in a PKS module [13]. This was tested in the KR domain in module 4 of the *epo* PKS that was introduced into the *M. xanthus* K111-72 strain that produced epothilone D as the major product. Three EpoD mutants, each containing either of the individual amino acid replacements Y2716F or G2566A, or both, were generated as *M. xanthus* K122-27, K122-28 and K122-29 strains, respectively. Another mutant, *M. xanthus* K122-30, was generated by deletion of the entire KR4 domain (2314 to 2826) and replaced with a 17 amino acid linker created from homology analysis employing PKS modules containing only KS, AT, and ACP domains. The strains K122-27 and K122-28, each carrying a single point mutation in *epo* KR4, produced a mixture of epothilones that included epothilone D as the major product (approximately 1~2 μg/ml) and the unexpected compound 11,12-dehydro-12,13-dihydro-13-oxoepothilone D (**5**) (Fig. 2A) as the most abundant minor product, at approximately 10~20% of the level of epothilone D seen . On the other hand, both the double mutant K122-29 and the deletion mutant K122-30 produced only **5**, as well as a minor product whose mass spectrum was consistent with the expected compound 12,13-dihydro-13-oxoepothilone C (Fig. 2A: **6**). Neither epothilone D nor the other expected compound, the 12-methyl congener of **6**, were detected in fermentation broths screened by LC/MS.

It appears that a single mutation at the active site or within the NADPH binding site only impairs the KR activity to a limited extent and allows the domain to function normally. This result differs from a recent report in

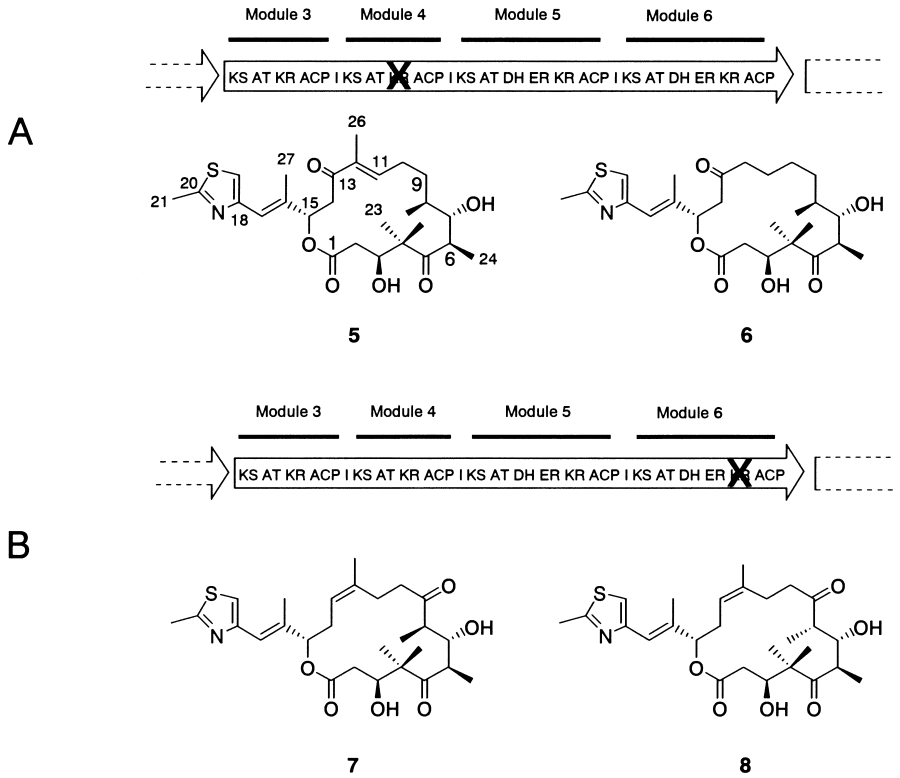

Fig. 2 Modifications of the KR4 (A) and KR6 (B) domains to produce epothilone analogues. The **X** placed over the KR4 or KR6 domains indicates that the domain has been inactivated as described in the text. Only modules 3~6 are shown. Abbreviations are described in Fig. 1.

which substitution of the active site Tyr residue with Phe resulted in complete inactivation of KR domain in DEBS module 6 [13]. Complete inactivation of *epo* KR4 was achieved in the double mutant or through deletion of the domain with the production of the expected compound, 12,13-dihydro-13-oxoepothilone C (**6**), consistent with its action during the fourth elongation cycle. Interestingly 12,13-dihydro-13-oxoepothilone D (the 12-methyl congener of **6**) was not found. Rather, the 12-methyl-13-oxo- compound that was produced contained an 11,12-double bond, mostly likely formed by migration of the 10,11-double bond generated during the fifth elongation cycle of nascent polyketide biosynthesis (after DH5-mediated dehydration but before ER5-mediated reduction) into conjugation with the keto group at C13. This explanation is supported by the isolation of the minor metabolite 10,11-dehydroepothilone D from the epothilone D producer strain [11]. The C-11 methylene of epothilone results from the full reduction of the keto group that is formed during biosynthesis of the polyketide backbone. Failure to reduce the ER5 domain in some instances suggests that ER5 is somewhat inefficient, hence the opportunity for migration of the 10,11-double bond in the nascent polyketide biosynthesis, driven by the acidity of H-12 in the KR4 mutants.

Production of 9-Oxoepothilone Analogues

A double mutant was generated by inactivation of the KR domain in module 6 by replacing the native Tyr residue with Phe (EpoD:Y6919F) and changing the putative NAD(P)H binding motif 6777-GGLGGLG-6783 to 6777-GALGGLG-6783. The resultant strain K39-164 produced the expected compound 9-oxoepothilone D (**7**) and 8-*epi*-9-oxoepothilone D (**8**) as the major products in approximately equal amounts, *ca.* 100~200 ng/ml (Fig. 2B). This result suggests that the double mutation in the KR6 domain eliminates all KR activity in this module. In addition, the production of two isomers indicates that the stereochemical control of the methyl-branched centers at C8 of epothilone likely either resides in the ER domain of module 6, or that the KR6 mutation aberrantly affects another domain, such as KS6, to abolish its function of stereochemical control. In addition to the major compounds **7** and **8**, several other compounds were isolated from a large-scale fermentation of strain K39-164. Most are related to 9-oxoepothilones C or D or their derivatives that have prematurely hydrolyzed

from the PKS enzyme before TE-catalyzed lactone formation [14]. Interestingly, 9-oxoepothilone C was not seen in the fermentation broths.

Attempts to produce a 9-hydroxyepothilone derivative through either inactivation of the DH6 domain or replacement of the DH6/ER6/KR6 encoding segment with various heterologous KR domains were not successful. Also, replacement of the entire DH/ER/KR6 segment with a linker similar to the strategy used to delete the KR4 domain resulted in the production of a 14-membered macrolide whose structure appeared to consistent with the biosynthesis having bypassed module 6 entirely (unpublished results). This explanation is supported by the isolation of the minor metabolite epothilone K from the epothilone production strain [3].

The presence of functional domains in type I modular PKSs generally insures production of a single product. The ER domain of module 5 in the wild type *epo* PKS is bypassed a small percentage of the time to produce small amounts of 10,11-didehydroepothilones C and D along with the major products, epothilones C and D. Normally, the 2,3-double bond introduced during the fifth elongation step of nascent polyketide biosynthesis is reduced by the ER5 domain to generate the 2,3-dimethylene centers that appear at the 10, 11 positions of the completed molecule. If it is bypassed, the double bond remains and the 10,11-didehydro- compounds are produced. In the *epo* PKS that contains an inactive KR4 domain, the nascent acyl chain to be acted on by ER5 has either the 2,3-didehydro-5-oxo- or 2,3-didehydro-4-methyl-5-oxo- functions, depending on whether the AT domain of module 4 utilizes malonyl CoA or methylmalonyl CoA as the extender unit during the fourth condensation cycle. If the former is presented to ER5, the double bond is reduced and 12,13-dihydro-13-oxo-epothilone C is produced. If the latter is presented to the ER, the double bond does not get reduced. Ultimately the 2,3-double bond shifts to the 3,4 position into conjugation with the 5-oxo function, most likely promoted by the ER domain, implying, therefore, that the ER5 domain of the *epo* PKS may act as a reductase in one case and as a 2,3-3,4-isomerase in another.

Experimental

Materials and General Methods

M. xanthus strains K25.25 and K111-72 were engineered to produce epothilones C and D as the major products by introduction of the epothilone gene cluster into the chromosome. Details of the constructions of these strains are presented elsewhere [7]. *M. xanthus* strains were grown in CYE (10% Casitone, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% Yeast extract, 50 mM HEPES [pH 7.6]) at 30°C or 32°C. For selection of galactose resistance in *M. xanthus*, cells were plated in 2.5 ml of CYE top agar and poured onto CYE plates containing 1% galactose.

To test for the production of epothilone analogues, cells were cultured in CTS (0.5% Casitone, 0.2% $MgSO_4 \cdot 7H_2O$, 50 mM HEPES [pH 7.6]) with 2% of the resin XAD-16 to absorb epothilones produced. Cells were initially grown in CYE medium to mid-log phase and a 5% inoculum was used to inoculate a flask containing the production medium. The cultures were grown at 30°C for 6 days.

Standard DNA protocols were used for growth, transformation and genetic manipulation of *Escherichia coli*. Electroporation of *M. xanthus* was described by Kashefi and Hartzell1 [5].

Construction of Replacement Cassettes

All changes (mutation, deletion, *etc.*) in the *epo* PKS were introduced by gene replacement in the *M. xanthus* host employing homologous recombination in segments upstream and downstream of the site to be modified. Hence, each altered segment was cloned in a delivery vector between an upstream and downstream segment of the *epo* PKS corresponding to the sites desired for homologous recombination.

For the construction of a KR deletion in module 4, the flanking regions of the KR4 domain were PCR amplified from the epothilone cluster with two pairs of oligonucleotide primers containing appropriate flanking restriction sites (shown in italics) (5′-ATG*AATTC*A-TGATGGCCCGAGCAGCG-3′ and 5′-AT*CTGCAG*CC-AGTACCGCTGCCGCTGCCA-3′; 5′-GC*TCTAGA*ACC-CGGAACTGGCGTGGCCTGT-3′ and 5′-GC*AGATCT*AC-CGCGTGAGGACACGGCCTT-3′). The product from the first PCR reaction was treated with *Eco*RI and *Pst*I and cloned into pLitmus28 (Stratagene) and the resulting plasmid was cut with *Pst*I and *Bam*HI and ligated with the second PCR product treated with *Xba*I and B*gl*II and a DNA duplex linker created from the following two oligonucleotides: 5′-GGCGCCGGCCAAGAGCGCCGCG-CCGGTCGGCGGGCCAGCCGGGGACGGGT-3′; 5′-CTAGACCCGTCCCCGGCTGGCCCGCCGACCGGCGC GGCGCTCTTGGCCGGCGCCTGCA*G*-3′ to generate plasmid pLit-KR4*. The replacement suicide vector pKOS122-30 was created by insertion of the 3 kb DNA segment containing the kanamycin resistance (*kanR*) and galactokinase (*galK*) genes from KG2 at the *Dra*I site of pLit-KR4*.

For the construction of KR4 mutation, a 4.5 kb *Rsr*II-*BsrD*I fragment of *epoD*, encoding the full KR domain and

its flanking region in module 4 was cloned in the vector pUC18 (Stratagene). The resulting plasmid was used as a template for site-directed mutagenesis employing the QuikChange kit with one of the following sets of PCR primers: 5′-CATAGCCAGGGTCCGT**T**CGCGGCGGCC-AACGCTTT-3′ and 5′-AAAGCGTTGGCCGCCGCG**A**-ACGGACCCTGGCTATG-3′, 5′-CGAGCTACCTGGTG-ACGGGAG**C**CCTCGGTGGGCTGGGCCTGAT-3′ and 5′-ATCAGGCCCAGCCCACCGAGG**G**CTCCCGTCACCAG GTAGCTCG-3′. The nucleotide changes resulted in the changes QSNYAAA… to … QSNFAAA… (Y2716F) and …GGLGGLG… to GALGGLG (G2566A) in the KR4 domain of EpoD. The KR4 mutations in the recovered clones pLit-KR4^{M} were confirmed by sequencing. The 3 kb DNA segment containing the *kanR* and *galK* genes from KG2 was inserted in the *Dra*I site of pLit-KR4^{M} to generate the delivery plasmids pKOS122-28 (Y2716F), pKOS122-27 (G2566A) and pKOS122-29 (Y2716F and G2566A).

For the construction of KR mutation in module 6, a 3.8 kb *Avr*II-*Kpn*I fragment of *epoD*, encoding the full KR domain and its flanking region in module 6 was cloned in the vector pUC19 (Stratagene). The resulting plasmid was used as a template for site-directed mutagenesis employing the QuikChange kit with one of the following sets of PCR primers: 5′-GCCGGGCCAGGGCAACT**T**CGCCGCGGC-CAACACGTT-3′ and 5′-AACGTGTTGGCCGCGGCG**A**-AGTTGCCCTGGCCCGGGC-3′, 5′-GCACCTACCTCG-GACCGGCG**C**TCTGGGTGGGCTCGGTCTGA-3′ and 5′-TCAGACCGAGCCCACCCAGA**G**CGCCGGTCACG-AGGTAGGTGC-3′. The two nucleotides change in sequence highlighted in bold results in the change of the corresponding polypeptide: from … QSNYAAA… to … QSNFAAA… (Y6919F) and from …TGGLGGLG… to TGALGGLG …(G6778A). The recovered clone pLit-KR6* containing the KR6 mutation was confirmed by sequencing. A 3 kb DNA segment containing the genes *kanR* and *galK* was inserted in the *Dra*I site of pLit-KR6* to generate the delivery plasmid pKOS39-164 (Y6919F and G6778A).

Construction of *M. xanthus* Strains that Produce Epothilone Analogues

The delivery vectors were introduced into the epothilone D producer *M. xanthus* strain K111-72 by electroporation employing selection for kanamycin resistance (50 μg/ml of kanamycin). This selects for integration of the delivery vector containing the *kanR* and *galK* genes along with the altered *epo* sequence in the chromosome and creates a duplication of the segments flanking the altered sequence. To isolate cells that had eliminated the duplicated segments, a second homologous recombination event was selected by growth on galactose plates. Growth on galactose requires elimination of the *galK* gene, which would take place after a second recombination event to result in excision of the plasmid from the chromosome and subsequent loss from the host, either from reversal of the original integration event or crossover in the other duplicated region. Galactose-resistant colonies were screened for the production of epothilones. Approximately one-half of the cultures were found to have reverted to the wild-type, the other half harbored the desired mutation and produced novel polyketides at titers ranging from 25 to 75% of the titer observed from the parent strain. All constructs were analyzed by PCR to ensure that the host had undergone the expected double crossovers.

Detection and Isolation of Epothilone Analogues

Small-scale fermentations were screened for production of epothilones by LC/MS on a Sciex API 100LC single quadrupole mass spectrometer equipped with an APCI source. Strains that produced metabolites with mass spectra consistent with new epothilones were selected and scaled up to provide material for complete characterization. After 6 to 7 days of growth in CTS with 2% XAD-16, the cultures were harvested by collection of the XAD-16 resin. The epothilone analogues were eluted from the resin with methanol and the eluate was partially concentrated. The resulting aqueous phase was extracted three times with 2 volumes of ethyl acetate. The combined organic extract was concentrated to dryness. The extract was subjected to silica gel column chromatography, eluting stepwise with 10%, 20%, 30%, 40% and 50% acetone in hexane. The individual compounds were purified further by HPLC employing a C-18 reversed phase column (10×250 mm 5 μ ODS-3 Inertsil, MetaChem) and eluting with a gradient of 30～100% acetonitrile/water over 45 minutes at a flow rate of 4 ml/minute with UV detection at 250 nm. These procedures enabled the purifications of 11,12-dehydro-12,13-dihydro-13-oxoepothilone D (**5**) and diastereomer 1 of 9-oxoepothilone D (**7** or **8**). Diastereomer 2 of 9-oxoepothilone D was purified as follows. XAD-16 adsorber resin, recovered from a 1000-liter fermentation, was washed with water and eluted with 78 : 22 methanol/water. The eluent was diluted with water to 1 : 1 methanol/water and loaded onto a column packed with 6 liters of HP20-SS (Mitsubishi) resin. The column was washed with 1 : 1 methanol/water and **7** and **8** were eluted using 75 : 25 methanol/water. The in-process pool was concentrated, diluted with 1 : 1 methanol/water and loaded onto a column packed with 0.5 liters of C18 reversed phase sorbent (Bakerbond, 40 micron, narrow pore). Elution with 62 : 38 methanol/water gave **7** and **8** as a mixture.

Chromatographic separation was accomplished using preparative HPLC (Varian, Polaris C18A, 2.1×25 cm, 10 micron, 35 : 65 acetonitrile/water). Evaporation of the corresponding product pools gave **7** and **8** as purified compounds.

Structural Elucidation of New Epothilones

HRMS spectra were measured on an Applied Biosystems Mariner time-of-flight mass spectrometer with a turbo-ionspray source in positive ion mode. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data were recorded in $CDCl_3$ solution at 300 K with a Bruker DRX 400 spectrometer equipped with a QNP z-axis gradient probe head. Chemical shifts in $CDCl_3$ solution were referred to δ 7.26 and 77.0 for ^{1}H and ^{13}C spectra, respectively. A series of two-dimensional NMR experiments was used to secure the structure of new epothilones. One-bond proton–carbon couplings were determined with HSQC experiments. Constant-time HMBC data were used to establish long-range proton–carbon couplings. Homonuclear proton couplings were measured with pure-absorption TOCSY or COSY experiments.

11,12-Dehydro-12,13-dihydro-13-oxoepothilone D (5)

ESI-TOF-HRMS: *m/z* 506.2576; calculated for $C_{27}H_{40}NO_6S$ $[M+H]^+$, *m/z* 506.2571. ^{1}H NMR δ 6.98 (s, H-19), 6.75 (t, *J*=7.0 Hz, H-11), 6.59 (s, H-17), 5.61 (dd, *J*=11.0, 2.5 Hz, H-15), 4.06 (dd, *J*=10.5, 2.5 Hz, H-3), 3.70 (dd, *J*=6.0, 4.5 Hz, H-7), 3.50 (dd, *J*=13.5 Hz, 10.0, Ha-14), 3.21 (qd, *J*=7.0, 4.5 Hz, H-6), 2.71 (s, H-21), 2.54 (m, Ha-10), 2.45 (m, Hb-14), 2.44 (dd, *J*=14.0, 10.5 Hz, Ha-2), 2.37 (m, Hb-10), 2.34 (dd, *J*=14.0, 2.5 Hz, Hb-2), 2.12 (d, *J*=1.0 Hz, H-27), 1.93 (m, Ha-9), 1.81 (s, H-26), 1.77 (m, H-8), 1.60 (m, Hb-9), 1.33 (s, H-22), 1.13 (d, *J*=7.0 Hz, H-24), 1.08 (d, *J*=7.0 Hz, H-25), 1.06 (s, H-23). ^{13}C NMR δ 220.8 (C-5), 199.8 (C-13), 164.8 (C-20), 151.9 (C-18), 169.9 (C-1), 145.0 (C-11), 138.2 (C-12), 137.4 (C-16), 119.6 (C-17), 116.4 (C-19), 77.3 (C-15), 74.5 (C-7), 73.2 (C-3), 52.8 (C-4), 42.9 (C-6), 40.9 (C-14), 38.9 (C-2), 33.5 (C-8), 29.0 (C-9), 25.6 (C-10), 20.7 (C-22), 20.2 (C-23), 19.1 (C-21), 17.8 (C-25), 15.5 (C-27),13.0 (C-24), 11.9 (C-26).

9-Oxoepothilone D, Diastereomer 1 (7 or 8)

ESI-TOF-HRMS: *m/z* 528.2409; calculated for $C_{27}H_{39}NO_6NaS$ $[M+Na]^+$, *m/z* 528.2391. ^{1}H NMR δ 6.97 (s, H-19), 6.56 (s, H-17), 5.18 (m, H-13), 5.17 (dd, *J*=9.5, 2.0 Hz, H-15), 4.12 (dd, *J*=9.0, 4.5 Hz, H-3), 3.81 (m, H-7), 3.27 (m, H-6), 2.78 (Ha-11), 2.77 (m, Ha-10), 2.77 (m, H-8), 2.72 (s, H-21), 2.72 (m, Ha-14), 2.56 (m, Hb-10), 2.15 (m, Hb-14), 2.09 (d, *J*=1.0 Hz, H-27), 1.85 (m, Hb-11), 1.70 (s, H-26), 1.29 (s, H-22), 1.33 (d, *J*=7.5 Hz, H-25), 1.21 (d, *J*=7.0 Hz, H-24), 1.12 (s, H-23). ^{13}C NMR δ 218.9 (C-5), 216.1 (C-9), 170.7 (C-1), 164.8 (C-20), 151.9 (C-18), 138.5 (C-16), 138.0 (C-12), 121.2 (C-13), 119.5 (C-17), 116.0 (C-19), 79.8 (C-15), 75.7 (C-7), 73.4 (C-3), 52.8 (C-4), 47.0 (C-8), 45.9 (C-6), 40.0 (C-10), 39.0 (C-2), 32.5 (C-14), 24.9 (C-11), 23.3 (C-26), 21.7 (C-22), 21.0 (C-23), 19.0 (C-21) 16.0 (C-25), 15.3 (C-27), 15.0 (C-24).

9-Oxoepothilone D, Diastereomer 2 (7 or 8)

ESI-TOF-HRMS: *m/z* 506.2598; calculated for $C_{27}H_{40}NO_6S$ $[M+H]^+$, *m/z* 506.2571. ^{1}H NMR δ 6.95 (s, H-19), 6.53 (br s, H-17), 5.27 (br t, *J*=5.5 Hz, H-15), 5.17 (br t, *J*=7.0, H-13), 4.19 (dd, *J*=8.5, 4.0 Hz, H-3), 3.99 (dd, *J*=10.0, 1.0 Hz, H-7), 2.84 (qd, *J*=7.0, 1.0, H-6), 2.76 (m, Ha-10), 2.67 (s, H-21), 2.64 (m, H-8), 2.52 (m, Hab-14), 2.48 (m, Ha-2), 2.44 (m, Hb-2), 2.40 (m, Hb-10), 2.37 (m, Ha-11), 2.18 (m, Hb-11), 2.04 (d, *J*=1.0 Hz, H-27), 1.65 (br s, H-26), 1.24 (d, *J*=6.5 Hz, H-25), 1.20 (s, H-23), 1.14 (d, *J*=7.0 Hz, H-24), 1.11 (s, H-22). ^{13}C NMR δ 221.6 (C-5), 212.4 (C-9), 170.1 (C-1), 164.8 (C-20), 152.0 (C-18), 137.4 (C-16), 136.8 (C-12), 120.7 (C-13), 119.6 (C-17), 115.9 (C-19), 78.2 (C-15), 72.7 (C-3), 71.3 (C-7), 52.5 (C-4), 50.8 (C-8), 43.2 (C-6), 39.2 (C-2), 37.6 (C-10), 31.2 (C-14), 25.3 (C-11), 23.0 (C-26), 21.4 (C-23), 20.6 (C-22), 19.0 (C-21), 15.7 (C-27), 14.1 (C-25), 10.9 (C-24).

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