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Remarkable stereospecific conjugate additions to the Hsp90 inhibitor celastrol

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Abstract

Celastrol, an important natural product and Hsp90 inhibitor with a wide range of biological and medical activities and broad use as a biological probe, acts by an as yet undetermined mode of action. It is known to undergo Michael additions with biological sulfur nucleophiles. Here it is demonstrated that nucleophiles add to the pharmacophore of celastrol in a remarkable stereospecific manner. Extensive characterization of the addition products have been obtained using NMR spectrometry, nuclear Overhauser effects, and density functional theory to determine facial selectivity and gain insight into the orbital interactions of the reactive centers. This stereospecificity of celastrol may be important to its protein target selectivity.

The natural product celastrol (**1**) (Figure 1) exhibits broad biological activity, including effects on protein homeostasis^{1–4} neurodegeneration,^{5–7} inflammation,^{8,9} and cancer.^{10–12} Recently, **1** has been identified as an inhibitor of heat shock protein 90 (Hsp90),¹³ an essential molecular chaperone with numerous cellular functions. Hsp90 is highly expressed in cancer cells and has a critical role in conformational maturation and activation of a wide range of signaling molecules that include pro-oncogenic proteins involved in cancer.¹⁴ Therefore, small molecule inhibitors targeting Hsp90 have great potential as anticancer therapeutics.^{15,16} Most of the inhibitors of this protein, such as geldanamycin¹⁷ and radicicol,¹⁸ interrupt the ATPase activity of Hsp90 resulting in enhanced degradation of the client proteins *via* the ubiquitin-proteasome pathway.¹⁹ However, since many of the ATP site binding compounds also exhibit undesirable side effects,²⁰ the identification of new types of inhibitors is of great importance.

Celastrol (**1**) is a non-ATP-competitive inhibitor^{13,21} representing a new therapeutic class and offering a new strategy for development of Hsp90 inhibitors with improved properties.

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Supporting Information. Synthetic procedures, ¹H-, ¹³C-, and n.O.e NMR spectra, detailed DFT calculations, and mass spectrum of glutathione adduct. This material is available free of charge via the Internet at <http://pubs.acs.org>. Instructions for Authors at (<http://pubs.acs.org/page/jacsat/submission/authors.html>).

Although studies from our laboratory¹ and others^{8,22,23} suggest a conjugate addition of cysteine residues to the quinone methide substructure to be the dominant mode of action, the exact mechanism is still far from understood, especially concerning the identity and nature of binding of **1** to its protein target(s). Studies confirmed that inhibition of the Hsp90 pathway is cysteine-mediated,^{22,24,25} but if, and how, **1** displays specificity and discriminates among the various potential targets remains elusive. Structurally, celastrol (**1**) belongs to a subgroup of D:A-friedo-nor-oleananes characterized by a hydroxyl group in the adjacent position to the carbonyl oxygen and a chromophore that extends over the A and B ring of the triterpenoid skeleton.^{26,27} These structural characteristics, along with steric effects and its conformation should influence its reactivity and stability. The hydroxyl group in the A ring also enhances the reactivity via intramolecular hydrogen bonding to the carbonyl. Extended conjugation into the B ring counteracts this reactivity. The quinone methide substructure of **1** incorporates three potentially electrophilic positions (C-2, C-4, and C-6), of which position C-4 appears to be too hindered to be susceptible to nucleophilic addition, and attack at C-6 is expected to be favored over C-2 as a result of the increased thermodynamic stability achieved by aromatization of the ring.

Here we investigate the reactivity and chemistry of the purported pharmacophore¹ of **1**, namely, the fused AB ring system. The reaction of celastrol with various nucleophiles was studied by 1D- and 2D-NMR spectroscopy and *in silico* analysis. We report studies that unequivocally establish that nucleophilic attack on celastrol is regioselective at the 1,6 electrophilic position and that this reaction possesses remarkable stereospecificity. Utilizing extensive NMR spectroscopy and theoretical calculations we establish that nucleophilic attack is favored exclusively at the β -face (as defined in Rose *et al.*²⁸ with the nucleophile approach *syn* to the β -C9 methyl). Literature precedent²⁹ involving the addition of acetate to the C-6 position of pristimerin, the methyl ester of celastrol, proposes α -facial addition, using the rationalization that the sterically congested β -face, having most of the angular methyl groups, especially at C-9, on that face, would preclude attack from an incoming nucleophile, although no experimental evidence was reported. Here we offer evidence that nucleophilic addition to celastrol and related quinone methide structures is to the β -face.

Celastrol (**1**) undergoes reduction with various hydride reagents exclusively at the 1,6-position, generating prochiral dihydrocelastrol (**2**)³⁰ (Figure 2) with complete discoloration, characteristic of reduction of the quinone methide substructure. We envisioned utilizing this reaction to introduce a radio-label for subsequent biological studies without altering the parent structure. Consequently, a model reaction with sodium borodeuteride was conducted, wherein we anticipated incorporation of ~50% deuterium. Remarkably, deuterium addition was observed to occur exclusively to the β -face of the C-6 center to provide deuteriohydroxycelestrol (**3**). When comparison is made to dihydrocelastrol (**2**) (Table S1, supporting information) it can be seen that the chemical shift of C-6H α (α -face proton) of **2** occurs at δ 3.28 as a doublet of doublets with the expected splitting occurring from the C-7 hydrogen and the enantiotopic C-6H β (β -face proton). Deuteriohydroxycelestrol (**3**) shows a slight upfield shift for the C-6H α proton to δ 3.25 because of the adjacent deuterium. What is surprising, however, is the doublet splitting pattern with the C-7 proton, suggesting stereospecific addition of the nucleophile to one face as opposed to the expected non-stereoselective addition, which would give rise to a doublet of doublets, as observed in **2**. Furthermore, as expected from the principle of microscopic reversibility, aerial oxidation of **3** back to **1** resulted in complete loss of deuterium. Intrigued by this observation, we conducted extensive n.O.e studies (Figure 3) to investigate the generality of the absolute stereochemistry of the addition. No n.O.e. effect was observed for the C-6 hydrogen at δ 3.25 with the C-9 methyl group occupying the β -face, suggesting that the hydrogen was facing 'down' (the α -face), and, therefore, nucleophilic addition occurred from the 'top' β -face.

However, numerous factors, such as intraatomic distance and the steric effect of the C-9 methyl group toward top face addition, demanded further investigation.

Further evidence that nucleophilic addition was exclusive to one face and that the face in question was the 'top' β -face was sought. Hence, dihydroxycelastrol (**2**) was subjected to n.O.e irradiation (Figure 3) of the δ 3.28 dd and δ 2.99 d signals (Table S1, Supporting Information) of the two enantiotopic protons at the C-6 carbon atom to determine if they interacted with another proton. An enhancement of the C-9 methyl signal at δ 1.27 was observed upon selective irradiation of the δ 2.99 signal (assigned, on this basis, as the β -face C-6 proton), and no enhancement of the C-9 methyl was observed upon irradiation of the δ 3.28 signal. These results provide conclusive evidence that a) the addition of hydride occurs stereospecifically to the β -face of celastrol and b) the intramolecular distance between the C-6 and C-9 centers is appropriate for analysis by n.O.e spectroscopy.

To demonstrate that the observed facial selectivity of nucleophiles is an inherent property of celastrol, we investigated a variety of nucleophilic reactions comprising hard and soft carbon, nitrogen, and sulfur nucleophiles. In all cases the experiments were conducted utilizing an excess of nucleophilic reagent to encourage the formation of both possible isomers. Initial studies with carbon nucleophiles established that **1** was inert to reactions with a large excess (~10 equiv) of methyl-magnesium bromide and methyllithium because hard organometallic nucleophiles act as bases, leading to deprotonation of the enol functionality, as confirmed by NMR spectrometry. This, in effect, renders celastrol poorly electrophilic and inert toward nucleophilic attack. Successful Michael addition of a carbon nucleophile was achieved, however, with a nitroalkane to form nitromethyl adduct **4**. The nitronate anion, generated *in situ* from nitromethane and tetra-*n*-butylammonium fluoride (TBAF), was employed; it is known that fluoride ions catalyze Michael additions of nitroalkanes to electron-deficient alkenes.³¹ Extensive spectroscopic analysis of **4** confirmed the exclusive stereospecific addition of the nitronate anion to the C-6 carbon of **1**. The signals of interest, the C-6 proton, and adjacent diastereotopic methylene protons from the newly introduced nucleophile, were observed as distinct signals devoid of overlap at δ 4.27, δ 4.12, and δ 4.54, respectively (Table S1, Supporting Information). The use of n.O.e irradiation again provided unequivocal evidence of addition of the nucleophile to the β -face (Figure 3). As expected, no enhancement of the C-9 methyl protons was observed upon irradiation of the C-6 proton, or *vice versa*, confirming the α -face orientation of the C-6 proton. Furthermore, irradiation of the signal corresponding to one of the diastereotopic methylene protons (δ 4.12) resulted in an enhancement of the C-9 methyl protons, confirming its position on the β -face.

Nitrogen nucleophiles were found to be inert to reaction with celastrol (**1**). A range of amines (primary and secondary, alkyl and aryl substituted) and reaction conditions (excess amine reagent, base strength) all failed to furnish an addition product. Other studies from our laboratory to convert the carboxylic acid functionality of **1** to various amides (including the nucleophilic amino acids lysine and histidine) also supported this observation.

Of critical interest to the potential biological mode of action of **1** is the stereoselectivity of sulfur nucleophiles, such as cysteine residues in biological milieu, which is the reaction purported¹ to be essential for its biological activity. To the best of our knowledge, previous studies have not reported on stereo-selectivity of this addition. The *N*-acetylcysteine adduct of **1** (compound **5**) was synthesized and subjected to n.O.e irradiation (Figure 3). Irradiation of the δ 4.70 doublet of the C-6 proton failed to cause enhancement of the C-9 methyl protons, indicating an α -facial orientation for the C-6 proton. This was confirmed by irradiation of the δ 3.37 diastereotopic methylene proton, which clearly displayed an n.O.e. to the C-9 methyl protons signal. These results provide conclusive evidence for β -face

addition of *N*-acetylcysteine to celastrol, which is a model for the addition of cysteine nucleophiles contained in proteins that add to **1**. To achieve a closer approximation to intracellular conditions, we also investigated the reaction of **1** with glutathione in buffered conditions (pH 7.5). Although mass spectrometric analysis (Supporting Information) showed the generation of the expected C-6 adduct, NMR spectrometry proved futile because of a combination of poor solubility and diminished and complex signals.

We postulate that soft nucleophiles are more reactive in nucleophilic additions to celastrol because of favorable orbital interactions. These nucleophiles possess large diffuse orbitals for interaction with the accepting orbitals of celastrol (**1**). In comparison, the highly polarized nature of hard, basic nucleophiles results instead in deprotonation of the celastrol hydroxyl functionality.

In an attempt to rationalize these observations and suppositions regarding a high degree of orbital involvement, which result in facial selectivity, comprehensive theoretical calculations were performed. Density functional theory (DFT) calculations were performed on **1** and one of the nucleophiles (the nitronate anion) using Jaguar 7.8 implemented in the Schrodinger 9.1 package.³² The B3LYP functional with 6-31G** basis set was utilized for optimizing the compounds along with the computation of Fukui functions. The Fukui indices are known to be reactivity indices, and they give the information about which atoms in a molecule have a larger tendency to either lose or accept an electron or, in other words, which atoms are more prone to undergo a nucleophilic or an electrophilic attack. The molecule undergoing nucleophilic or an electrophilic attack has the tendency of becoming polarized in the presence of an external field or upon change in electron density. The idea behind Fukui's indices calculations lies in the realm of conceptual DFT. Mineva et.al.³³ carried out selectivity studies of compounds used as reagents in organic synthesis by using density functional local reactivity indices. They computed orbital Fukui indices from Kohn-Sham orbitals and also from the atomic resolved hardness matrix to study the reaction mechanism along with regioselectivity.³³ The analysis of the Fukui indices is primarily done for the HOMO and LUMO of the reactants. Jaguar computes the atomic Fukui indices according to the method developed by Perez *et.al.*^{34,35} These indices are based on Fukui functions, which are partial derivatives of the electron and spin density with respect to a change in either the electron count or the unpaired spin count. Atomic Fukui indices are an attempt to quantify the anticipated reactive center in a molecule. Each integrated index has two subscripts, N or S, which represent the electron density and spin density, respectively. Usually f_{-NN} indices are of interest, because the other indices require a change in either the spin density or the spin multiplicity. The f_{-NN} of HOMO indices are related to $f(-)$ Fukui function, and f_{-NN} LUMO indices are related to $f(+)$ Fukui function.

We have computed all the Fukui functions for structure **1** and the nucleophilic nitronate anion. It was observed that the f_{-NN} LUMO of **1** at position C-6 (#10 in DFT number, see SI) has the highest index (0.2116), depicting that nucleophilic attack has to be at this center. For the nucleophile, the f_{-NN} HOMO index of the carbanion center is highest and found to be 0.6124 (see SI). This clearly indicates that the carbanionic center of the nucleophile is expected to attack carbon C-6 of **1**, consistent with the hard-soft-acid-base (HSAB) principle.³⁶ After identifying the reactive centers in **1** and the nucleophile, we analyzed the HOMO contour of the nucleophile and LUMO contour of **1**. The orbital analysis clearly indicates the stereo-specific conjugate addition of **1** (Figure 4A). We then computed the final product obtained in the above reaction. To identify the stable product, we carried out DFT calculations for both geometries of **4** (Figure 4B). The DFT optimized geometries reveal a 11.6 kcal/mol (see SI) energy difference between the two structures in favor of **4 β** . This clearly indicates that the reaction site at C-6 produces a much more stable product (**4 β**) by β -facial attack, which is consistent with the experimental observations detailed above.

It is conceivable that this stereospecific addition may be an important factor in selectivity of reaction of protein targets with celastrol; binding to some proteins containing cysteine residues might not lead to reaction because the cysteine residue is oriented on the wrong face of bound celastrol. The notion of facial selectivity is well known in biology; an analogy is with reactions catalyzed by pyridine nucleotide (NAD(P)⁺ and NAD(P)H)-dependent enzymes. These cofactors are prochiral and bind to their respective enzymes in a specific orientation that favors stereospecific addition of hydride to either the substrate from NAD(P)H or to NAD⁺ by the substrate, the stereochemistry of which is determined by the orientation of bound substrate.³⁷

In conclusion, celastrol (**1**) is believed to undergo formation of a Michael adduct with its intracellular target(s),^{17–20} and this interaction appears to be crucial for a number of biological properties that this natural product exhibits. We have demonstrated unprecedented stereospecificity in the regioselective Michael reaction of celastrol with a range of nucleophiles, especially soft nucleophiles such as biologically-relevant cysteine and glutathione. This, heretofore, unknown chemistry of celastrol may help to unlock its selectivity for protein targets and mode of biological action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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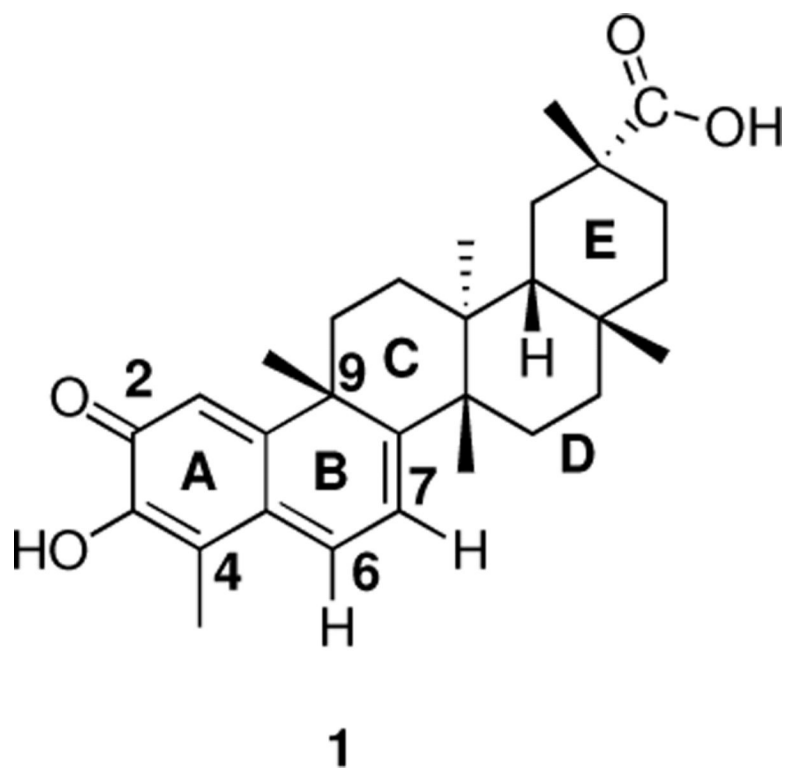


Figure 1.
Structure of Celastrol

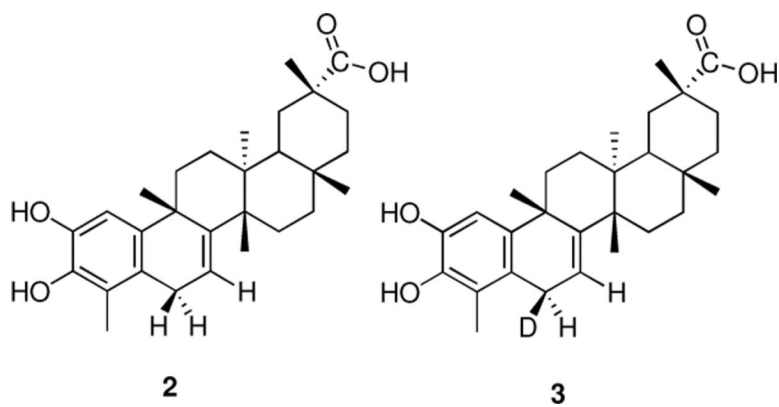


Figure 2.
Structures of dihydroxycelastrol (2) and deuteriohydroxycelastrol (3).

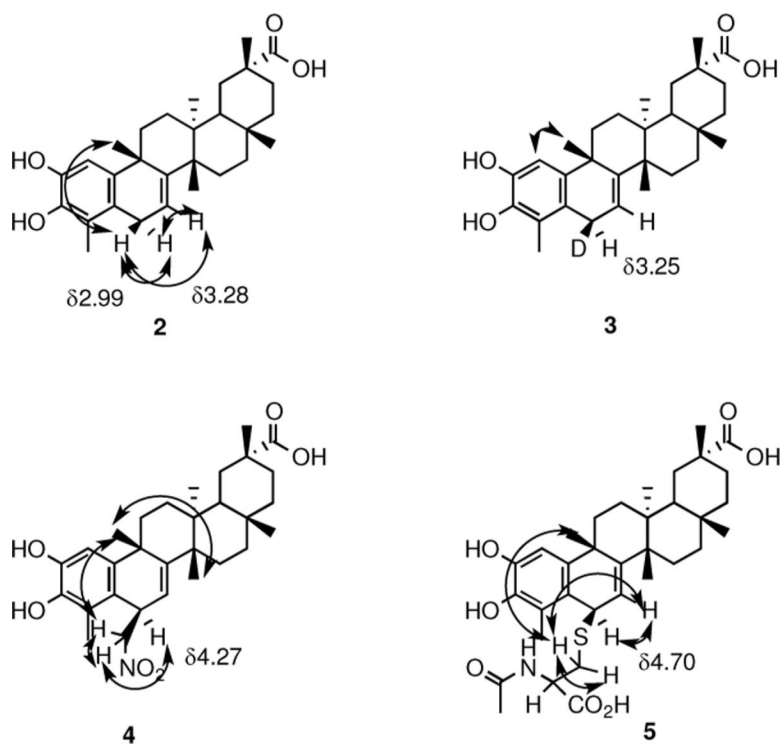


Figure 3.
Nuclear Overhauser effect couplings observed for β -face addition

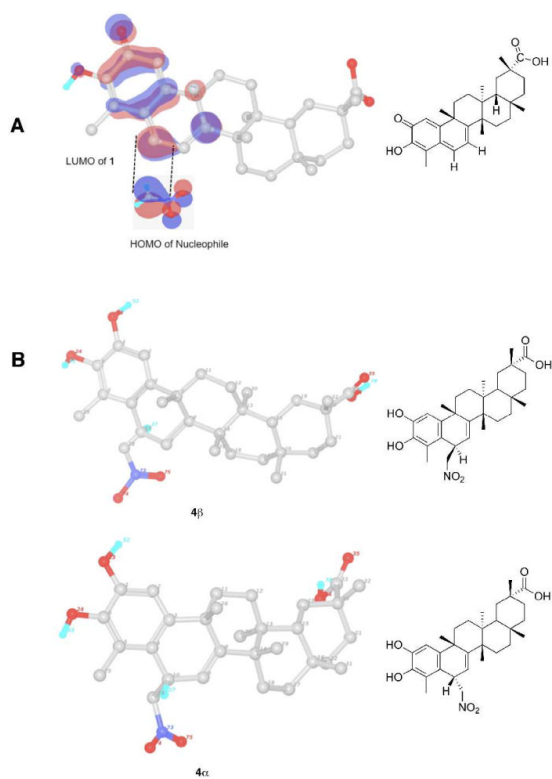


Figure 4.

A; Orbital analysis of celastrol (**1**) clearly shows the LUMO (red) positioned on the β -face. The nitronate anion HOMO (blue) attacks at the upper face. Blue - highest electron density; red - lowest electron density. B; Density functional theory (DFT) minimized structures of 4β and 4α .