Bioorganic & Medicinal Chemistry 16 (2008) 7573–7581

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and cellular effects of cycloterpenals: Cyclohexadienal-based activators of neurite outgrowth ☆

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ARTICLE INFO

Article history:
Received 10 December 2007
Revised 5 July 2008
Accepted 14 July 2008
Available online 19 July 2008

Dedicated to Prof. Robert S. H. Liu at the University of Hawaii, Manoa in honor of his 70th birthday

Keywords:
Terpene
Proline
Imine addition
Neurite outgrowth

ABSTRACT

An unusual class of diterpenoid natural products, 'cycloterpenals' (with a central cyclohexadienal core), that arise in nature by condensation of retinoids and other isoprenes, have been isolated from a variety of organisms including marine sponges as well as from the human eye. A milk whey protein has also demonstrated the formation of a cycloterpenal derived from β-ionylidineacetaldehyde. Here, we generate a synthetic library of these molecules where we detail reaction conditions required to effect cross condensation of α,β-unsaturated aldehydes as opposed to homodimerization. The ability of this class of molecules to activate neurite outgrowth activity is reported.

1. Introduction

The carotenoids are responsible for such processes as light absorption as well as for the inactivation of free radicals.[1] The retinoids, for example, the natural and synthetic derivatives of vitamin A, serve as chromophores of the visual transduction system (Fig. 1).[2] Retinoids also exhibit a variety of other biological activities. They are used extensively in dermatology for a variety of conditions, show activity as cancer preventative agents, and are potent modulators of growth and differentiation.[3–5] In this regard, *all-trans* retinoic acid (**1**) has been shown to differentiate embryonic stem cells into a neuron-like phenotype.[6] It has also been employed as a cancer chemotherapeutic in treatment of promyelocytic leukemia by causing terminal differentiation of the malignant cells[4] while retinoic acid analogs (TTNPB **2**, azulenic compounds **3**, and **4**) have been shown to possess biological activity as cancer chemopreventative agents.[2]

Interestingly, self-condensation of polyene aldehydes has been observed in nature resulting in terpenes with cyclodimeric structures. We have defined these molecules as cycloterpenals as they are composed of a cyclohexadiene core formed by the condensation of two terpene aldehydes. Cyclocitral (**5**), for example, was

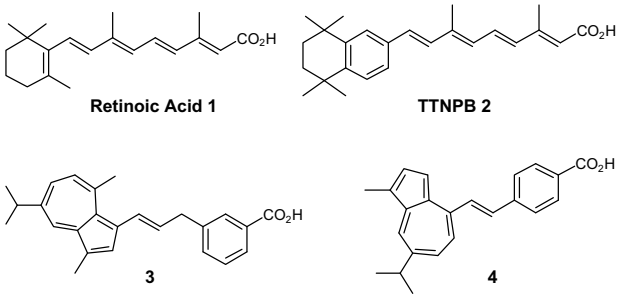

Figure 1. Structures of representative retinoid compounds.

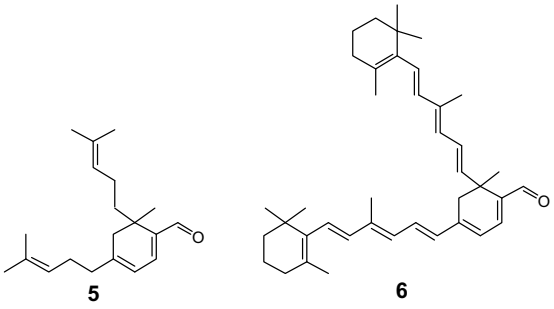

Figure 2. Structures of citral and retinal self-condensation products.

☆ A U.S. patent on this work has been filed (US-2007-0232813-A1). The patent application publication can be accessed at http://www.uspto.gov/patft/.

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doi:10.1016/j.bmc.2008.07.030

recently isolated from the North Sea bryozoan *Flustra foliacea* and shown to exhibit antibiotic activity (Fig. 2),[7–9] while cycloretinal (**6**) has been suggested as a contributor of age-related macular degeneration.[10,11] Furthermore, incubation of the milk whey protein beta-lactoglobulin with β-ionylideneacetaldehyde has been shown to result in cyclohexadienal formation.[12]

On these bases we broadly speculate that some of these cycloterpenals or their structurally and functionally related analogs could be important in developmental processes such as neurite outgrowth. To begin to address this issue, we designed a synthetic library of cyclohexadienals and tested the composite library (ring-fused heterodimers and homodimers, Fig. 3)[13] against PC12 cells,

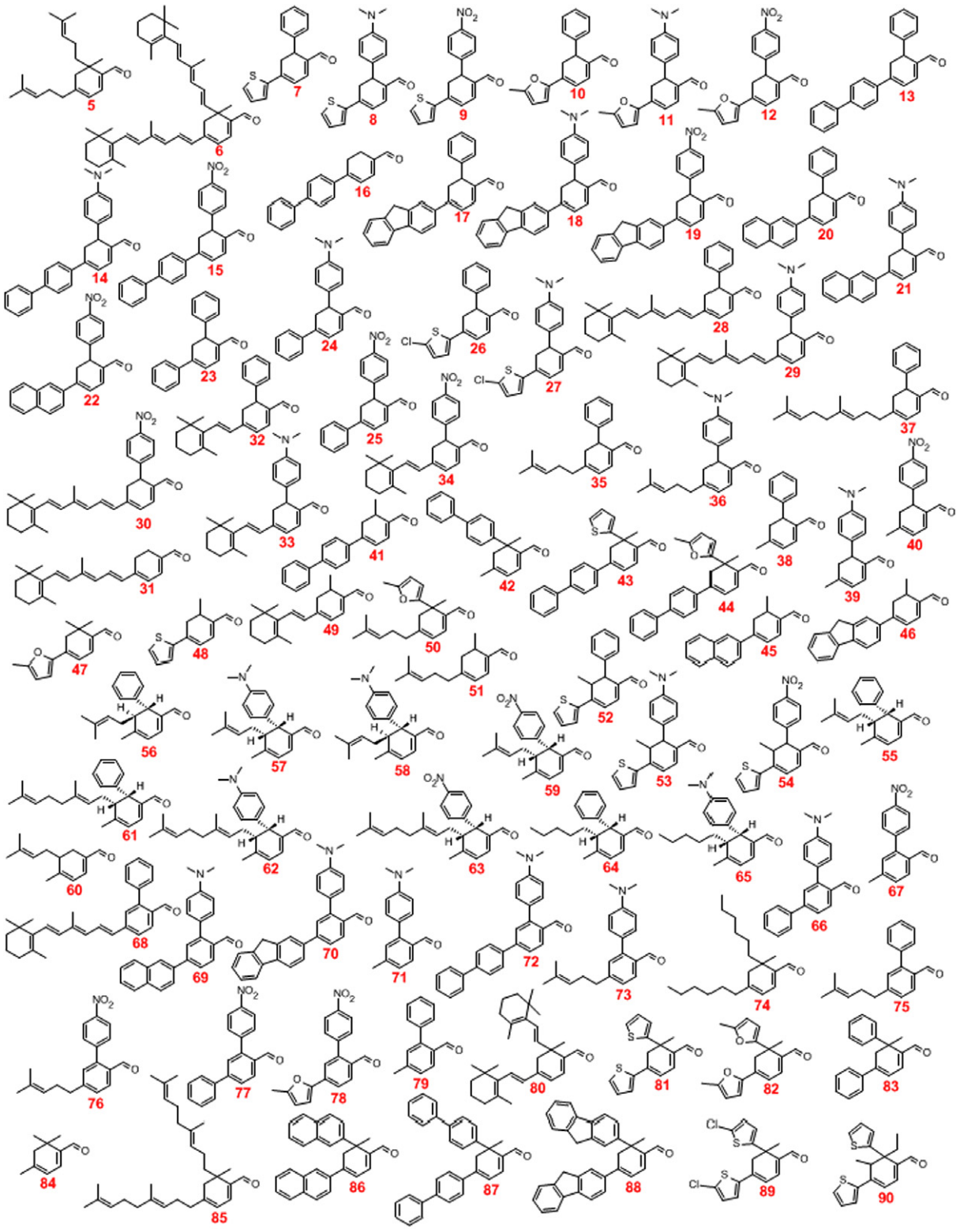


Figure 3. Chemical structures of the cycloterpenal-based compound library.

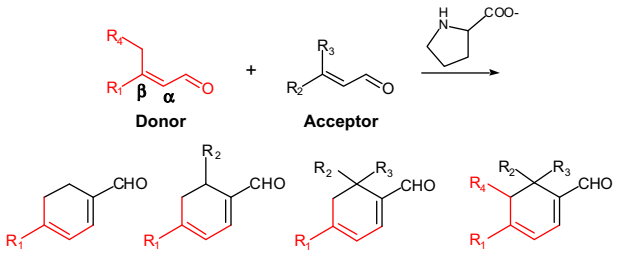


Figure 4. Substituted heterodimeric cyclohexadienals generated by proline-mediated condensation of α,β-unsaturated aldehydes.

where promising effects were observed on neurite outgrowth. In some instances, involving aromatic substitutions to the central ring, the cyclohexadiene ring system could be aromatized to give fluorescent molecules. In the future, some of these molecules or their respective derivatives might find use as fluorescent probes in protein target identification of a particular cyclohexadienal.

2. Results and discussion

2.1. Synthesis of asymmetric cyclohexadienals

The cycloterpenal-based compound library (Fig. 3) was generated by proline-promoted condensation of α,β-unsaturated aldehydes giving mono-, di-, tri-, and tetra-substituted cyclohexadienals (Fig. 4). The reaction involves condensation between a donor and acceptor aldehyde, where donor molecules require the presence of either a β-methyl- or β-methylene group. Acceptor molecules exhibit a wider degree of flexibility, the single constraint owing to the presence of an α,β-unsaturated aldehyde. As demonstrated by NMR, MS, and LC/MS analyses (see Supplemental material), the reaction proceeds by complete conversion of aldehyde to that of the Schiff base followed by a Diels–Alder or Michael-like imine addition.[13]

2.2. Reaction optimization and scope

Here, we detail optimal reaction conditions to favor heterodimer formation over competing homodimerization,[13] which is inherent to the reaction. The reaction is, therefore, not amenable to a one-pot strategy (unlike homodimerization) as minimal yields are obtained under such conditions. Moreover, we illustrate the scope of the reaction by invoking substrates that vary in functionality and architecture.

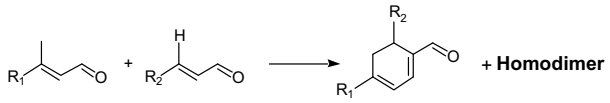
2.2.1. Catalysis involving a single β-methyl substituted substrate

Employing a one-pot strategy, reaction of 3-thiophen-2-yl-but-2-enal with 4-(dimethyl-amino)cinnamaldehyde (in a 1:1 ratio) gave a 5% and 43% yield, respectively, of heterodimer **8** to homodimer **81** (Table 1). Likewise, skewing the ratio in favor of the acceptor aldehyde substrate over that of the β-methyl substituted aldehyde donor in a one-pot format gave poor yields of the heterodimer **8**. Therefore, we implemented this reaction as a model system and optimized its conditions in an effort to define a generalizable set of reaction conditions to maximize heterodimer yields.

As α,β-unsaturated aldehydes lacking a β-methyl group fail to self-dimerize (confirmed through ESI mass spectroscopy) and the reaction can be envisioned to proceed through formation of a Schiff base,[13,14] we examined the notion of pre-forming the proline Schiff base of the acceptor aldehyde (as monitored by TLC, the Schiff base appears at the base-line with concomitant loss of the starting aldehyde) prior to the addition of the donor aldehyde. Intuitively, this should minimize formation of the cyclic homodimer **81**, while maximizing heterodimer **8** yields. To our satisfaction, an overall enhancement in reaction yield (from 5% to 64%) was indeed obtained. Such a strategy greatly facilitates reaction yields except in the case of 4-nitrocinnamaldehyde, which undergoes unknown side reactions after 13 min as detected by NMR (see Supplemental material). With this method, we were able to limit the formation of the homodimer product anywhere from 1% to 14% dependent upon the substrate.

Other factors found to influence the cross-condensation reaction included substrate (donor to acceptor) ratios and temperature effects. For instance, varying the ratio of donor to that of the acceptor (in a 1:1, 1:1.5, 1:2, and 1:3), at a set catalyst ratio (1:1.5 substrate to catalyst ratio, shown to be optimal from previous studies), gave surprisingly higher yields at 1:1 (21.4%) and 1:1.5 (64%) molar concentrations. At 1:2 and 1:3 molar concentrations, the overall heterodimer yields were 14.0% and 11.2%, respectively. Based upon these findings, we employed 1 (donor): 1.5 (acceptor): 3.8 (L-proline) molar concentration ratios to effect synthesis of the remainder of the heterodimers involving a single β-methylenic substrate.

Table 1
Reactions involving a single β-methyl substituted aldehyde



R1[a]	R2[a]	Comp #	Yield[b] (%)	ee[c] (%)
Thienyl	Benzyl	**7**	48	56
Thienyl	Dimethylamino-benzyl	**8**	64	53
Thienyl	Nitrobenzyl	**9**	23	49
CH_3 furanyl	Benzyl	**10**	48	63
CH_3 furanyl	Dimethylamino-benzyl	**11**	62	72
CH_3 furanyl	Nitrobenzyl	**12**	22	50
Biphenyl	Benzyl	**13**	45	52
Biphenyl	Dimethylamino-benzyl	**14**	75	62
Biphenyl	Nitrobenzyl	**15**	16	53
Biphenyl	H–	**16**	57	–
Fluorenyl	Benzyl	**17**	47	43
Fluorenyl	Dimethylamino-benzyl	**18**	59	62
Fluorenyl	Nitrobenzyl	**19**	22	49
Naphthyl	Benzyl	**20**	50	57
Naphthyl	Dimethylamino-benzyl	**21**	58	54
Naphthyl	Nitrobenzyl	**22**	34	56
Benzyl	Benzyl	**23**	53	64
Benzyl	Dimethylamino-benzyl	**24**	64	54
Benzyl	Nitrobenzyl	**25**	34	51
2-Cl thienyl	Benzyl	**26**	42	36
2-Cl thienyl	Dimethylamino-benzyl	**27**	56	41
Retinyl	Benzyl	**28**	55	56
Retinyl	Dimethylamino-benzyl	**29**	64	56
Retinyl	Nitrobenzyl	**30**	22	50
Retinyl	H–	**31**	51	–
β-Ionyl	Benzyl	**32**	47	56
β-Ionyl	Dimethylamino-benzyl	**33**	57	59
β-Ionyl	Nitrobenzyl	**34**	35	53
Dimethylallyl	Benzyl	**35**	12	52
Dimethylallyl	Dimethylamino-benzyl	**36**	5	40
Farnesenyl	Benzyl	**37**	15	62
Methyl	Benzyl	**38**	60	56
Methyl	Dimethylamino-benzyl	**39**	63	48
Methyl	Nitrobenzyl	**40**	47	54

[a] Reaction conditions: 1 (donor): 1.5 (acceptor): 3.8 (L-proline); the acceptor was dissolved in ethanol, reacted with 2 h with L-proline to which donor was added and stirred at room temperature for 16–24 h.
[b] Isolated yield.
[c] Determined by $Pr(hfc)_3$ chiral shift reagent.

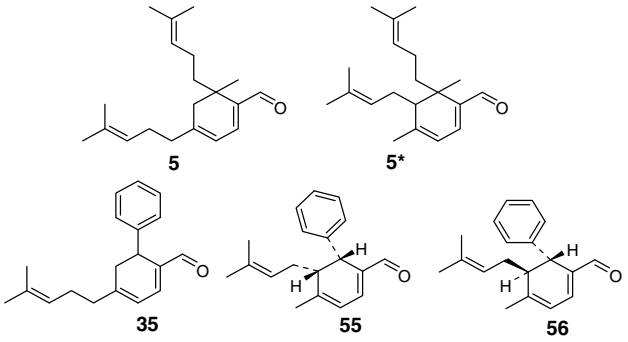


Figure 5. Schematic representation of products resulting from reaction of cinnamaldehyde with citral.

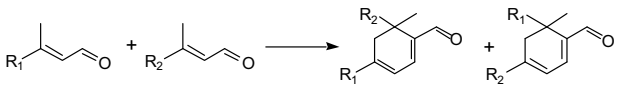
Temperature effects on the reaction paralleled that observed with self-condensation reactions,[13] whereby product yields were highest at ambient temperature versus −20 to 0 °C or 50 to 100 °C. In all cases the concentration of catalyst, L-proline, in these reactions was held constant at 3.8 equivalents since these amounts were shown to give the greatest extents of conversion in homodimer reactions.[13] Use of L-proline (3.8 equivalents) maintains a substrate to catalyst ratio of 1:1.5, where substrate equals donor plus aldehyde.

Utilizing these optimized conditions, we investigated the overall scope of the reaction as illustrated in Table 1. In most cases the heterodimers were generated in modest yields. The lowest yields were obtained with heterodimers bearing dimethylallyl-**35** and -**36** or farnesenyl-**37** substituents giving reaction yields in the 5–15% range, which presumably is attributed to the flexibility of the side chains and competing side reactions (Fig. 5). Conversely, the highest yields were achieved with heterodimers bearing rigid substituents (aromatic or other conjugated systems). Such trends are also observed with homodimerization.[13] As expected, in almost all cases the cross-condensation reaction was favored by electron donating groups as opposed to electron withdrawing substituents. Homodimer yields ranged from 1% to 14% in each instance.

2.2.2. Catalysis between two β-methyl substituted substrates

Proline-promoted synthesis between two β-methylenic substrates would be expected to yield a mixture of substituted cyclohexadienals, corresponding to two homodimeric and two heterodimeric products, respectively (Table 2). To achieve these cross-condensation reactions, reaction conditions were modified slightly. A 1:1 ratio of each aldehyde substrate and 3 equivalents of L-proline were optimal. Skewing the ratio (1:2) only enhanced homodimer formation of the more prevalent substrate.

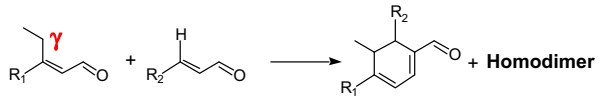
Table 2
Reaction between two β-methyl substituted aldehydes



R1[a]	R2[a]	Compound	Yield[b] (%)	ee[c] (%)
Biphenyl	Methyl	**41**	49	55
Methyl	Biphenyl	**42**	18	57
Biphenyl	Thienyl	**43**	14	54
Biphenyl	CH_3 furanyl	**44**	29	50
Naphthyl	H–	**45**	57	49
Fluorenyl	H–	**46**	67	48
CH_3furanyl	Methyl	**47**	40	52
Thienyl	H–	**48**	45	47
β-Ionyl	H–	**49**	39	44
Dimethylallyl	CH_3 furanyl	**50**	23	46
Dimethylallyl	H–	**51**	59	46

[a] Reaction conditions: 1 (donor): 1 (acceptor): 3 (L-proline) was dissolved in ethanol and stirred at room temperature for 16–24 h.
[b] Isolated yield.
[c] Determined by $Pr(hfc)_3$ chiral shift reagent.

Table 3
Reaction with β-ethyl substituted donor aldehydes



[a]R1	[a]R2	Compound	[b]Yield (%)	[c]ee (%)
Thienyl	Benzyl	**52**	49	52
Thienyl	Dimethylamino benzyl	**53**	50	50
Thienyl	Nitrobenzyl	**54**	25	51

[a] Reaction conditions: 1 (donor): 1.5 (acceptor): 3.8 (L-proline); the acceptor was dissolved in ethanol, reacted for 2 h to which donor was added and stirred at room temperature for 16–24 h.
[b] Isolated yield.
[c] Determined by $Pr(hfc)_3$ chiral shift reagent.

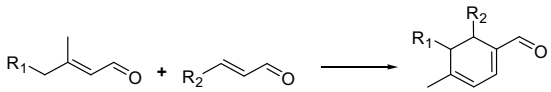
Implementing these optimized conditions, we examined the generality of the reaction. Sterics appeared to govern these cross condensations, as the only heterodimer product formed gave the bulkiest substituent at C-4 and the least hindered group at C-6 with the exception of **41** and **42**, in which both heterodimeric products were formed. Likewise, reaction of citral and crotonaldehyde gave rise to tri-substituted cyclohexadienal **51**, with the dimethylallyl-linkage at C-5 (Fig. 3). Reaction yields ranged from 14% to as high as 67%, with the highest yields arising from condensation with an acceptor aldehyde where R_2 is H– or CH_3–. On the contrary, the lowest yields were obtained from reaction with an acceptor aldehyde where R_2 is thienyl or methylfuranyl. While one might expect high level of homodimer to be formed with this one-pot strategy, the isolated yields ranged from 3% to 27% in each case.

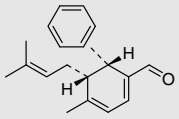
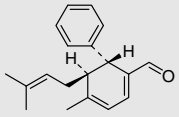
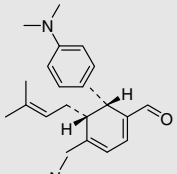
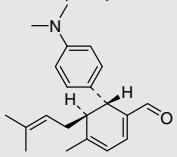
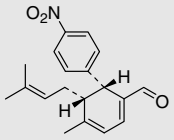
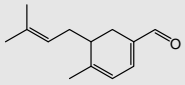
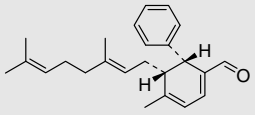
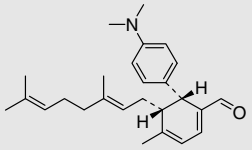
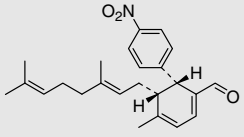
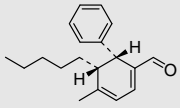
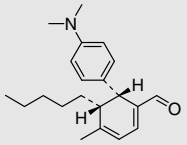
2.2.3. Catalysis effected by the β-methylene group of the donor aldehyde

Interestingly, catalysis is not limited to reaction with β-methyl substituted aldehydes. Substitution at C-5 of the cyclohexadienal was readily observed as a result of deprotonation occurring from the γ-methylene position of the donor aldehyde (Table 3). For instance, use of a β-ethyl substituted donor aldehyde gives methyl-substitution at C-5 of the tri-substituted cyclohexadienal. In this regard, reaction of 3-thiophen-2-yl-pent-2-enal with three different α,β-unsaturated aldehydes resulted in the formation of heterodimers shown in Table 3 as the major product (yields ranging from 25% to 50%) and approximately 10% homodimer in each instance. While it might be conceivable to generate the seven membered ring system by proton abstraction of the δ-methyl group, formation was not observed. Reaction conditions were the same as detailed previously and similar yields and ee's were observed.

Likewise, reacting cinnamaldehyde with citral gave three heterodimer products (Fig. 5), the expected di-substituted product **35** and two tri-substituted cyclohexadienals, *cis* and *trans* diastereomers (**55** and **56**), respectively. Previous work has actually shown that formation of homodimer **5*** can be favored (95:5) in the presence of strong base, NaH resulting in a mixture of homodimers.[15] The compounds were assigned on the basis of computational analyses and NMR spectroscopy (as described in Supplemental material). While we limited the formation of self-condensation products (**5** and **5***) by allowing cinnamaldehyde to go to complete Schiff base formation prior to the addition of citral, homodimer **5** was still observed in 9% yield. Heterodimers **35**, **55**, and **56** were formed in 12%, 25%, and 4% yield, respectively. As with

Table 4



R1 and R2[a]	Product	Compound	Yield[b] (%)	ee[c] (%)
R1 dimethylallyl R2 benzyl		**55**	25	53
		**56**	4	62
R1 dimethylallyl R2 dimethyl aminobenzyl		**57**	24[d]	33
		**58**		41
R1 dimethylallyl R2 nitrobenzyl		**59**	18	44
R1 dimethylallyl R2 H-		**60**	24[d]	45
R1 farnesenyl R2 benzyl		**61**	18	63
R1 farnesenyl R2 dimethyl aminobenzyl		**62**	45	61
R1 farnesenyl R2 nitrobenzyl		**63**	23	55
R1 pentyl R2 benzyl		**64**	29	57
R1 pentyl R2 dimethyl aminobenzyl		**65**	31	55

[a] Reaction conditions: 1 (donor): 1.5 (acceptor): 3.8 (L-proline) the acceptor was dissolved in ethanol, reacted for 2 h to which donor was added and stirred at room temperature for 16–24 h.
[b] Isolated yield.
[c] Determined by $Pr(hfc)_3$ chiral shift reagent.
[d] Diastereomers were inseparable by HPLC.

previous systems, these reactions favored substituents with electron donating groups as opposed to those bearing electron withdrawing substituents. In this regard, reactions involving nitrobenzyl groups gave correspondingly lower yields than those involving benzyl or dimethylamino-benzyl substituents.

These β-methylene driven reactions were also demonstrated with farnesenal, β-methyl substituted nonenal in combination with acrolein, cinnamaldehyde and its derivatives (Table 4). In most instances only the *cis* product was formed; citral was purchased as a mixture of *cis* and *trans* isomers while all others were obtained as a single (*all-trans*) isomeric form.

2.3. Aromatization and fluorescence

Interestingly, phenyl substituted cyclohexadienals (derived from cinnamaldehyde and its substituted analogs) were found to spontaneously aromatize (Fig. 6) to give fluorescent molecules (over a period of one to several days) in about 5–15% conversion. Aromatization could also be invoked directly by reaction with potassium permanganate in quantitative yields.[16] With further experimentation, some of these molecules or their respective derivatives might therefore find use as fluorescent probes to identify the protein binding partner of a particular cyclohexadienal. Quantum yields[20] are provided here (Table 5), as measured in dichloromethane and methanol, for a subset of the molecules including those that were most fluorescent as judged by exposure to UV light (structures of additional compounds isolated are provided as Supplemental material). In all cases, fluorescence of molecules was quenched in methanol. The highest quantum yields were obtained for compounds **71** and **73** (in dichloromethane), where the substituent at C-4 is not conjugated to the central aromatic ring. While nitro-substituted phenyl systems fluoresce mildly, the nitro-group was shown to significantly quench the quantum yield.

2.4. Effects on neurite outgrowth and general cytotoxicity

Given the demonstrated ability of retinoic acid and its analogs to modulate growth and cellular differentiation and the ability of a milk whey protein to generate cyclo-β-ional, the cycloterpenal-based library of compounds was evaluated for their ability to stimulate neurite outgrowth in a PC12 assay. PC12 cells are a pluripotent cell line that was originally cloned in 1976 from a transplantable rat pheochromocytoma and exhibits the ability to differentiate into neurons. The PC12 assay is a widely implemented method of identifying compounds that promote neurite outgrowth or survival.[17]

The PC12 cells were plated onto collagen Type IV plates at 10,000 cells/well, allowed to adhere for 24 h, followed by the addition of compound at concentrations of 100, 10 and 1 μg/mL, respectively. The cells were monitored over a period of two weeks, where the most pronounced effects were observed at about one week of exposure. From our library of cyclohexadienals, two heterodimers (**44** and **21**, Fig. 7) were able to successfully stimulate neurite outgrowth, while one homodimer **74**[17] supported the development of neural extensions. Therefore, neurite outgrowth activity appears to be associated with aromatic substituents or extended hydrocarbon chains. Intriguingly, biphenyl groups have been thought to serve as mimics of protein alpha helices.[18] Long aliphatic chains, on the other hand, are capable of inserting into lipid bilayers.

To assess the degree to which stereochemistry dictates phenotype in the neurite outgrowth assay, dimers **21**, **44**, and **74** were derivatized into their corresponding menthylhydrazone derivatives[19] to allow separation of the stereoisomers by HPLC. Following HPLC purification, the compounds were each hydrolyzed giving each aldehyde in enantiomerically pure form. Upon reanalysis in the PC12 assay, the *R*-configuration was shown to be responsible for neurite outgrowth activity while the *S*-configuration exhibited no activity (see Supplemental material for photos). In the future, it will be interesting to establish whether heterodimers **21** and **44** share the same target as the aliphatic homodimer **74**.

Table 5
Quantum yields of a representative set of fluorescent cyclohexadienals

Entry	% Conv.	CH_2Cl_2			MeOH		
		λ_{abs} (nm)	λ_{em} (nm)	φ[a]	λ_{abs} (nm)	λ_{em} (nm)	φ[a]
66	10	368	490	0.025	360	503	0.008
67	14	368	559	0.016	368	413	0.002
68	7	368	474	0.042	374	598	0.056
69	13	374	520	0.112	384	434	0.005
70	14	368	526	0.046	368	497	0.044
71	11	363	502	0.699	354	396	0.002
72	15	368	519	0.088	368	515	0.005
73	8	368	501	0.312	368	522	0.010

[a] Measured as specified previously.[20] Quinine sulfate was used as standard for quantum yield measurements (φ = 0.55 in H_2SO_4).

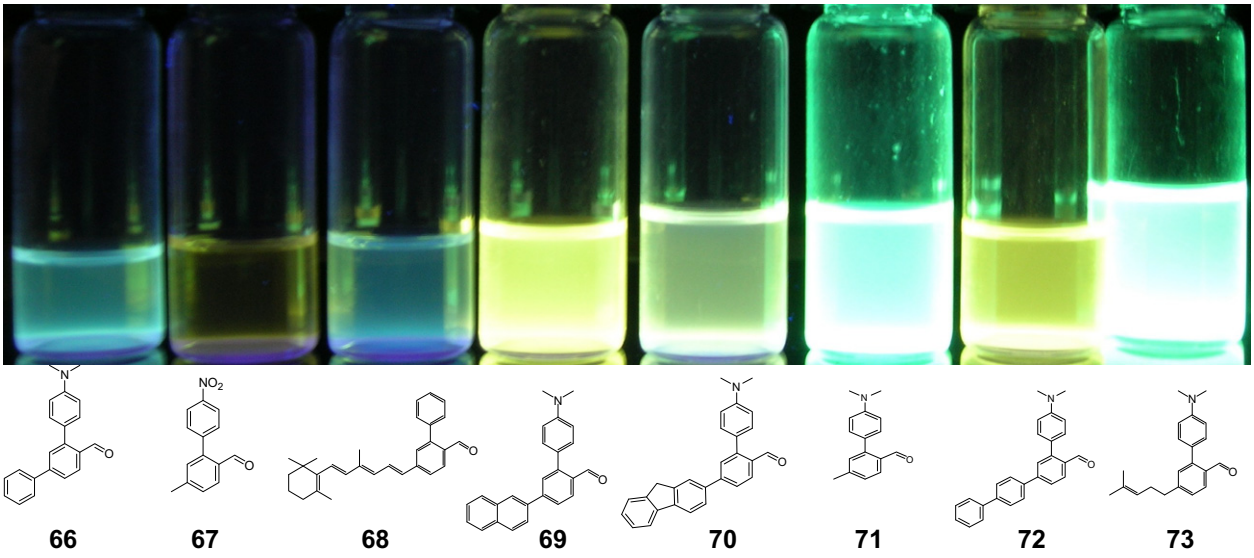


Figure 6. Representative set of fluorescing aromatized heterodimers.

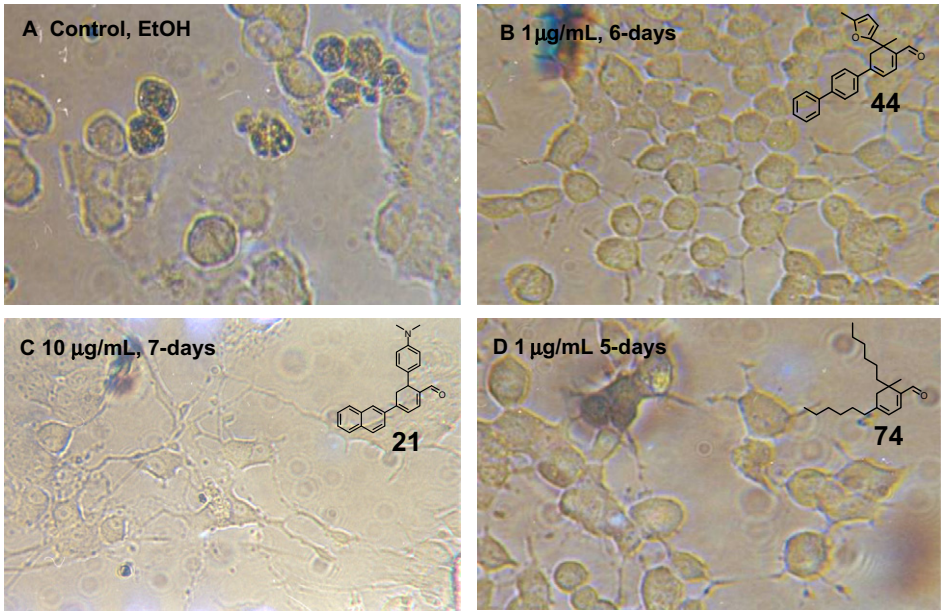


Figure 7. Stimulation of neurite outgrowth by cyclohexadienal heterodimers and homodimers: (A) Control, cells treated with absolute EtOH; (B) methylfuranyl, biphenyl-cyclohexadienal, 1 µg/mL at 6 days (C) dimethylamino-phenyl, naphthyl-cyclohexadienal; 10 µg/mL 7 days (D) nonenal-cyclohexadienal, 1 µg/mL 5 days.

As citral dimer **5** has been reported to possess antibiotic activity,[7–9] we also surveyed our cyclohexadienal library of compounds against bacterial strains (*Escherichia coli*, *Bacillus subtilis*) and yeast (*Saccharomyces cerevisiae*). See Supplemental material for graphical representation of data. All compounds were tested at three concentrations, 100, 10, and 1 µg/mL. In most cases, moderate to no inhibition was observed, where compounds exhibited MIC values >100 µg/mL. Three compounds (**71, 59**, and **43**) were reasonably active against *B. subtilis* with MIC values within the 10–25 µg/mL range. Against Jurkats, a human T-cell Leukemia cell line, while the effects were again moderate for the majority of compounds tested, a few (**44, 15, 19**, and **27**) demonstrated complete inhibition of growth at 1 µg/mL.

2.5. Conclusion

The retinoids have achieved prominence for their therapeutic benefits ranging from their ability to treat acne and skin blemishes to their ability to differentiate stem cells.[1–6] An unusual class of diterpenoid natural products, 'cycloterpenals', have been isolated from a variety of organisms including marine sponges and the human eye.[7–11] Moreover, incubation of the milk whey protein beta-lactoglobulin with β-ionylideneacetaldehyde has been shown to give cyclo-β-ional (a C-30 ring-fused dimer).[12] As such it is plausible that some of these cycloterpenals or their structurally and functionally related analogs could be important in developmental processes such as neurite outgrowth.

In this investigation, we implemented a proline-promoted condensation reaction that enabled diversification of the cycloterpenal skeleton to give a library of molecules with varying degrees of substitution. Specific reaction conditions required to favor cross condensation as opposed to self-condensation were detailed. In the case of aromatic substituents, the cyclohexadiene ring system could be aromatized to give fluorescent molecules, which in the long term could lead to use of these molecules as probes in protein target identification of a particular cyclohexadienal.

The phenotypic effects of these cycloterpenal-based molecules were examined in a PC12 assay, where dramatic effects were observed on neurite outgrowth. These results suggest that molecules with this cyclohexadienal motif might have applications in neurite regeneration, which plays a significant role in the treatment of neurodegenerative diseases or CNS injuries.

Experiments are now underway to investigate the enzymatic reaction in the biosynthesis of cycloterpenals.

3. Experimental

3.1. General methods

Fresh THF was dried by passing it through an MBRAUN solvent purification system and stored over molecular sieves. All other solvents and reagents were purchased and used without further purification. Chemical shifts for ^{1}H and ^{13}C NMR spectra are reported in ppm referenced to TMS (0 ppm) and coupling constants are reported in hertz (Hz). MS spectra were recorded on an API QSTAR PULSAR (ES) apparatus. IR spectra were recorded with a FT-IR spectrometer. Chromatographic separations were achieved by flash silica chromatography (silica gel 60 mesh, EMD Biosciences). Determination of ee's was achieved by $Pr(hfc)_3$ chemical shift reagent. β-Methylenic aldehydes (**101**–**111**) were synthesized by reduction of their corresponding nitriles (**91**–**100**),[21] which were derived from their respective methyl-ketone analogs.[22] In all cases, the *all-trans* isomer was utilized except in the case of citral which was purchased as a mixture. Ring-fused homodimers were prepared by self-condensation of the β-methylenic aldehyde substrates.[13]

3.2. Organisms

PC12 neuronal cells (CRL 1721) and *B. subtilis* strains 6633 were obtained from the American Type Culture Collection, Rockville, MD. The yeast *S. cerevisiae* wild-type strain [#404; BY4741; MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0] was obtained from Dr. Michael Kladde at the Department of Biochemistry, Texas A&M University. The *E. coli* strain DH10B was obtained from Prof. Dennis Gross, Texas A&M University, Department of Plant Pathology.

3.3. Neurite outgrowth assay

PC12 cells were maintained in 100 mm Collagen Type IV dishes at 37 °C in a 5% CO_2/air temperature. The cell culture medium

consisted of RPMI 1640, with 10% heat-inactivated horse serum and 5% fetal bovine serum. Cells were removed from the culture flasks by flushing with fresh culture medium. Cells were then transferred to a 15 mL falcon tube and centrifuged for 15 min at 1000 rpm. The medium was removed and the cells were resuspended in fresh culture medium. The cells were counted with a hemocytometer and replated in a 96-well Collagen Type IV plate at 10,000 cells/well with enough fresh medium to bring the volume to 200 μL. The cells were allowed to adhere for 24 h prior to the addition of compound. Each cyclohexadienal compound was examined at three concentrations in duplicate, to give final concentrations of 100, 10, and 1 μg/mL, respectively. Each well was visually monitored over a period of 2 weeks for neurite outgrowth and intercellular connections. Fresh medium was added when necessary. Photographs were taken with an Olympus SP-310 camera equipped with a modified lens piece that fits over the microscope eyepiece.

3.4. Cytotoxicity assay against the human T-cell leukemia, Jurkat cell line

Jurkat cells were maintained in 75 cm^2 culture flasks at 37 °C under a 5% CO_2 atmosphere. The cell culture medium consisted of RPMI 1640 with 10% fetal bovine serum. Cells were harvested by centrifugation (20 min at 1000 rpm), resuspended in fresh culture medium and counted on a hemocytometer. The cells (1 mL) were arrayed (200,000 cells/well) in 48-well plates to which compound was added to give final concentrations of 100, 10, and 1 μg/mL, respectively. Cells were incubated for 24 h, transferred to 1.5 mL eppendorf tubes and centrifuged for 15 min at 14,000 rpm. The medium was removed and the cell pellet resuspended in 1 mL of phosphate buffer saline (PBS) to wash the cells. The cells were again centrifuged for 15 min at 14,000 rpm. The PBS was removed and the cell pellet resuspended in a lysis buffer. The lysed cells were then placed in a black 96-well plate and analyzed using a Bio-Tek fluorometer to measure relative fluorescence.

3.5. Anti-bacterial and anti-fungal assays

Microbes (*Saccharomyces cerevisiae*, *Escherichia coli*, *Bacillus subtilis* 6633 and *Bacillus subtilis* 21332) were cultured and maintained on agar plates (see Supplemental material for media conditions). A single colony was used to inoculate 3 mL of culture medium and allowed to grow for 16 h. The cells (500 μL) were diluted with 50 mL of fresh medium to an OD of 0.1 and aliquoted into a 96-well plate (100 μL/well). The assays were set up in duplicate and the compounds were tested to give final concentrations of 100, 10, and 1 μg/mL. Plates were incubated (*E. coli* and *B. subtilis*, 37 °C; yeast, 30 °C) and shaken (250 rpm) for 16 h, and cell density was (OD) measured with a Bio-Tek microplate reader.

3.6. 4-(5-Methyl-furan-2-yl)-6-phenyl-cyclohexa-1,3-dienecarbaldehyde (Table 1, Entry 10): general procedure for the proline-mediated condensation of the α,β-unsaturated aldehydes

The α,β-unsaturated compound cinnamaldehyde (33.0 mg, 1.5 equivalents) was dissolved in 200 proof ethanol (8 mL) with 3.8 equivalents (72.0 mg) of L-proline. After approximately 2 h, at which point Schiff base formation is complete as monitored via thin layer chromatography (TLC), one equivalent of β-methylenic aldehyde 3-(5-methylfuran-2-yl)-but-2-enal (25 mg, 1 equivalent) was added to the reaction mixture and stirred at ambient temperatures for 18 h. The reaction was monitored by TLC. Upon completion, the reaction was quenched by the addition of water (20 mL), extracted with a 1:1 ethyl acetate/hexanes solution (3 × 50 mL), and was washed with brine. The organic layer was dried over magnesium sulfate (MgSO4) and concentrated under vacuum. The crude product was purified by column chromatography (10% ethyl acetate/hexanes) to yield 12.7 mg of the final product (48% yield) as a red oil. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C), δ = 9.54 (s, 1H), 7.12–7.28 (m, 5H), 7.10 (d, J = 6.3 Hz, 1H), 6.68 (dd, J = 2.6, 6.2 Hz, 1H), 6.45 (d, J = 3.3 Hz, 1H), 6.05–6.06 (m, 1H), 4.19 (dd, J = 1.8, 9.3 Hz, 1H), 2.87–3.09 (m, 2H), 2.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) δ = 191.9 (CH), 155.2 (C), 151.9 (C), 144.1 (CH), 142.9 (C), 137.9 (C), 134.0 (C), 128.7 (2 × CH), 127.4 (2 × CH), 127.0 (CH), 115.1 (CH), 112.6 (CH), 108.9 (CH), 34.0 (CH), 31.5 (CH2), 14.2 (CH3); IR (neat) ν 3012, 2962, 2941, 2326, 1735, 1431, 1365, 1229, 1211, 1206, 913 cm^{-1}; HRMS (ESI) for $C_{18}H_{16}O_2Li$ $(M+Li)^+$: calcd 271.1310, found 271.1365.

3.7. 4-(9H-fluoren-2-yl)-6-methylcyclohexa-1,3-dienecarbaldehyde (Table 2, Entry 46): general procedure for the proline-mediated condensation of two β-methylenic aldehydes

In a dried 100 mL flask, 3-(9H-fluoren-2-yl)-but-2-enal (100 mg, 1 equivalent) and crotonaldehyde (30 mg, 1 equivalent) were dissolved in 15 mL of 200 proof ethanol. L-Proline (300 mg, 3 equivalents) was added to facilitate the reaction. The reaction mixture was stirred for 16 h at room temperature before being quenched by the addition of 20 mL of water. The reaction mixture was extracted with a 1:1 ethyl acetate/hexanes solution (3 × 50 mL) and was washed with brine. The organic layer was dried over magnesium sulfate ($MgSO_4$) and concentrated under vacuum. The crude product was purified by column chromatography (10% ethyl acetate/hexanes) to yield 57.6 mg of the final product (67% yield) as a green solid. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C), δ = 9.56 (s, 1H), 7.77–7.90 (m, 2H), 7.55–7.59 (m, 2H), 7.25–7.44 (m, 3H), 6.90 (d, J = 6.0 Hz, 1H), 6.65 (dd, J = 2.7, 6.0 Hz, 1H), 3.93 (s, 2H), 3.05–3.15 (m, 1H), 2.95 (dq, J = 2.7, 8.4 Hz, 1H), 2.76–2.78 (m, 1H), 1.04 (d, J = 7.2 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) δ = 192.6 (CH), 145.7 (C), 143.9 (C), 143.8 (C), 142.8 (C), 142.7 (CH), 141.6 (C), 141.3 (C), 138.7 (C), 127.4 (CH), 127.2 (CH), 125.4 (CH), 125.0 (CH), 122.6 (CH), 120.4 (CH), 120.2 (CH), 119.1 (CH), 37.2 (CH_2), 33.9 (CH_2), 24.4 (CH), 18.1 (CH_3); IR (neat) ν 3053, 2962, 2925, 2868, 1733, 1663, 1546, 1423, 1265, 1183, 1046, 825, 733, 703 cm^{-1} HRMS (ESI) for $C_{21}H_{18}O$ $(M+H)^+$: calcd 287.1436, found 287.1463.

3.8. Determination of quantum yields

Relative quantum yields[20] were obtained with a photon counting spectrofluorometer equipped with a photomultiplier tube, which is sensitive up to 850–900 nm. The slit width was 0.5 nm for both excitation and emission sources. The quantum yield was calculated with the following equation:

$$\phi_{\mathbf{x}} = \phi_{\mathbf{st}}(\mathbf{I_x}/\mathbf{I_{st}})(\mathbf{A_{st}}/\mathbf{A_x})(\eta_{\mathbf{x}}^2/\eta_{\mathbf{st}}^2)$$

where the x subscript denotes unknown, st denotes standard, φ_{st} is the reported quantum yield of the standard, **I** is the integrated emission spectra, **A** is the absorbance at the excitation wavelength and **η** is the refractive index of the solvent used. Quinine sulfate (φ = 0.55 in 0.1 M H_2SO_4) was used as the standard.

3.9. Synthesis of 2-isopropyl-5-methylcyclohexyl hydrazinecarboxylate (112)[19]

To synthesize 2-isopropyl-5-methylcyclohexyl hydrazinecarboxylate **112**, ethyl 2-isopropyl-5-methylcyclohexyl carbonate was first prepared by reacting 25.0 g (1 equivalent) of L-menthol with 75 mL of ethyl chloroformate (7.3 equivalents) in the presence of pyridine (1 mL). The reaction was refluxed for 48 h before the light

brown product was poured over ice followed by dilution with 20 mL of water. The mixture was then extracted with ethyl ether (5 × 100 mL) with the organic layers combined, dried over $MgSO_4$, and concentrated under vacuum. Assuming 100% conversion of the ethyl 2-isopropyl-5-methylcyclohexyl carbonate, 75 mL of 2-ethoxy ethanol was used to solubilize the starting material. Once completely dissolved, a twofold excess of hydrazine monohydrate (20 mL) was being added, and then the reaction mixture was poured over ice and extracted with ethyl ether (5 × 100 mL) with the organic layers combined, dried over $MgSO_4$, and concentrated under vacuum. Once the ethyl ether was evaporated, 50 mL of hexanes was added and the solution was heated and left at −20 °C for 48 h in which white fluffy crystals were formed and were subsequently collected via vacuum filtration. The recrystallization was performed three more times yielding 12.7 g (51% yield) of **112**. ^{1}H NMR (300 MHz, $CDCl_3$, 25 °C), δ = 6.14 (s, 1H), 4.56 (dt, J = 4.5, 21.9 Hz, 1H), 3.74 (s, 2H), 1.97–2.02 (m, 1H), 1.82–1.92 (m, 1H), 1.61–1.66 (m, 2H), 1.40–1.52 (m, 1H), 1.30 (t, J = 10.8 Hz, 1H), 1.00–1.10 (m, 1H), 0.91–0.99 (m, 2H), 0.85–0.89 (m, 6H), 0.75 (d, J = 6.9 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, 25 °C) δ = 159.0 (C), 75.7 (CH), 47.5 (CH), 41.6 (CH_2), 34.4 (CH_2), 31.6 (CH), 26.4 (CH), 23.7 (CH_2), 22.2 (CH_3), 21.0 (CH_3), 16.6 (CH_3); HRMS (ESI) for $C_{11}H_{22}N_2O_2$ $(M+H)^+$: calcd 215.1760, found 215.1752.

3.10. 2-isopropyl-5-methylcyclohexyl 2-((4,6-dihexyl-6-methylcyclohexa-1,3-dienyl)methylene) hydrazinecarboxylate (115): general procedure for the synthesis of the menthylhydrazones[19]

Homodimer 4,6-dihexyl-6-methylcyclohexa-1,3-dienecarbaldehyde **74** (0.100 g, 1 equivalent) was dissolved in 200 proof ethanol (20 mL) with 1.5 equivalents (0.111 g) of 2-isopropyl-5-methylcyclohexyl hydrazinecarboxylate **112** at room temperature. After 72 h, the reaction was extracted with 1:1 ethyl acetate/hexanes solution (2 × 100 mL), and was washed with brine. The organic layer was dried over magnesium sulfate ($MgSO_4$) and concentrated under vacuum. The crude material was passed through a silica plug with 10% ethyl acetate/hexanes as the mobile phase. The mixture was then subjected to high pressure liquid chromatography (HPLC) with a mobile phase of 96% ethyl acetate/4% hexanes with a flow rate of 1.5 mL/min. Two fractions were collected at 11.524 and 12.501 min corresponding to the *R* and *S* stereoisomers, respectively. HRMS (ESI) for $C_{31}H_{54}N_2O_2$ $(M+H)^+$: calcd 487.4264, found 487.4238.

3.11. General procedure for the conversion of 2-isopropyl-5-methylcyclohexyl 2-((4,6-dihexyl-6-methylcyclohexa-1,3-dienyl)methylene) hydrazinecarboxylate (115) back to 4,6-dihexyl-6-methylcyclohexa-1,3-dienecarbaldehyde (74)[19]

Once the *R* and *S* stereoisomers were purified, each was dissolved in 20 mL of 1:1 water/ethanol with 5 mL of 10% sulfuric acid and boiled for 2.5 h in which a color change from clear to pink occurred within 2 h. The reaction mixture was removed from the heat source and allowed to cool when it was then extracted with ethyl ether (2 × 25 mL). The organic layer was then dried with $MgSO_4$ and concentrated under vacuum. The crude mixture was then passed through a silica plug with a mobile phase of 10% ethyl acetate/hexanes. HRMS (ESI) for $C_{20}H_{34}O$ $(M+H)^+$: calcd 291.2688, found 291.2667.

Acknowledgments

We thank the Welch Foundation (A-1587), Texas A&M University, and the NIH CBI Training Program (T32 GM008523) for support of this work. We also thank Ms. Vanessa Santiago for mass spectrometry analyses, Jiney Jose for help with quantum yield determination, and Dr. Joseph Reibenspies for X-ray crystal structure determination.

Supplementary data

Experimental procedures, additional characterization data, 1H and 13C NMR spectra, X-ray data, LC/MS data, NMR studies on Schiff base formation are provided. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.07.030.

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