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Efficient syntheses and anti-cancer activity of xenortides A–D including *ent*/*epi*-stereoisomers†

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A one-pot, two-step, total synthesis of naturally occurring xenortides A, B, C and D, (Xens A–D) isolated from the bacterium *Xenorhabdus nematophila*, and an entire complementary set of stereoisomers, has been achieved. Compounds were synthesized utilizing an isocyanide-based Ugi 4-CR followed by facile *N*-Boc deprotection. The reaction sequence took advantage of the chiral pool of *N*-Boc protected amino acids (L-Leu/Val and D-Leu/Val) with aryl isocyanides, phenyl acetaldehyde and methylamine giving the desired Xens A–D (A and B >98% ee) and all subsequent stereoisomers in reasonable yields upon deprotection followed by separation of diastereomers. Also, detailed mechanistic insights for diastereoselectivity of (–)-Xen A, as a model in the Ugi 4-CR, has been described. Moreover, for the first time, this focused library was screened for cytotoxicity against a panel of epithelial cancer cell lines as well as normal cell lines with an MTT proliferation assay. The structure–activity relationship (SAR) study demonstrated that tryptamides Xen B and D were more active than phenylethylamides Xen A and C. Furthermore, (–)-Xen B (IC_{50} = 19–25 μM) and *ent*-(+)-Xen D (IC_{50} = 21–26 μM) gave the highest cytotoxicity and they were also found to be non-toxic toward normal cells. Importantly, the SAR results indicate that the stereochemistry at C_8 and C_{11} in (–)-Xen B and *ent*-(+)-Xen D play a critical role in cytotoxic activity.

Introduction

Cancer is the second leading cause of death globally and as reported by the World Health Organization (WHO), cancer was responsible for 8.8 million deaths in 2015.[1] Despite an overall positive progression in cancer chemotherapy, limited improvements in patient survival has been documented in many countries and death rates are increasing. Multidrug resistance, lack of selective chemotherapeutic drugs against target tumor cells and severe side effects are major limitations for the availability of anticancer agents.[2] The treatment of cancer has been a serious concern in public health and urgent development of novel classes of therapeutic agents or anticancer drugs with improved efficacy are in high demand.[3,4] Peptides, compared to other small organic molecules and proteinaceous immunotherapeutics, have several desirable properties such as high activity, low immunogenicity, good biocompatibility, and they are readily amenable to synthetic modifications.[5,6] As such, peptides have become promising molecules for anticancer agents.[7–9] It is therefore important to strategize an efficient synthetic approach that can eliminate any unwanted material during the process to find an optimal molecule consisting of the most appropriate structural conformation.[10]

It is well-known that secondary bacteria and fungi metabolites are valuable sources of chemotherapeutic agents.[11,12] Bacterial secondary metabolites, xenortides (Xens) A–D (Scheme 1), are a family of natural products extracted from the bacterium *Xenorhabdus nematophilus*.[13–15] (–)-Xen A and C consist of *N*-phenylethylamide derivatives of the dipeptide (*N*-Me-L-Leu and *N*-Me-L-Phe) and (*N*-Me-L-Val and *N*-Me-L-Phe), respectively. The counterparts, (–)-Xen B and D, consist of tryptamide derivatives of the dipeptide (*N*-Me-L-Leu and *N*-Me-L-Phe) and (*N*-Me-L-Val and *N*-Me-L-Phe) respectively. Recent observations by Reimer *et al.* suggest that these compounds have some anti-parasitic activities.[14] In view of the aforementioned biological activities of naturally occurring Xens A–D and our longstanding interest in eradicating cancer,[16–19] exploring the potential anticancer activity of these and derivative compounds, encouraged us to devise a facile synthetic approach for naturally occurring Xen compounds and a cohort of stereo-

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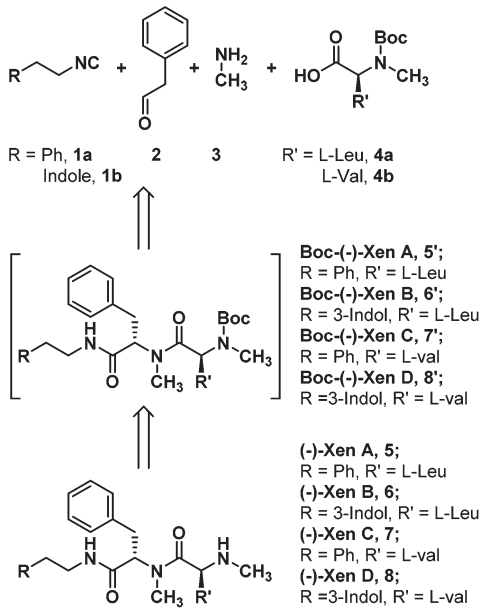


Scheme 1 Retrosynthetic approach to naturally occurring Xens A–D using an Ugi 4-CR (**1(a,b)** + **2** + **3** + **4(a,b)**).

isomers. Here within, we document our synthetic strategy to Xens A–D including all possible stereoisomers. Furthermore, we demonstrate compound bio-potential by examining anticancer activity of each isomer in several epithelial cancer cells. Moreover, this study represents the first anticancer activity report on the Xen family.

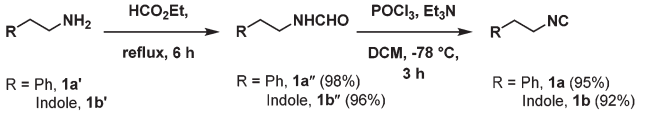
Recently, the synthesis of (−)-Xen D was reported by Reimer *et al.*[14] (−)-Xen D was synthesized *via* standard peptide coupling reaction conditions using several expensive peptide coupling reagents and multi-step syntheses giving rise to several purification procedures. Furthermore, the aforementioned strategy would not be easily accessible to a small library of derivatives as further synthetic manipulations would be required. These limitations encouraged us to devise a streamlined chemical route toward the synthesis of highly enantiopure Xens. To the best of our knowledge, there are no literature reports describing the synthesis of the complete set of Xen natural products and their *ent*/*epi*-stereoisomers. Moreover, we believe that the synthesis of highly enantiopure natural Xens A–D and their related stereoisomers may shed light on how the stereochemical properties of chiral, non-racemic Xens play out in anticancer studies, which have never been previously attempted. In the context of developing simple structural manipulations of the starting materials and atom-economical synthetic routes for the synthesis of Xens and all their stereoisomers, we designed an efficient and effective synthetic strategy employing an Ugi four component reaction (4-CR) process.[20–23] Our synthetic strategy, involving only two sequential steps, accomplished in a single pot, allows us to obtain the desired compounds in a highly stereoselective fashion using readily available commercial starting materials. As such, this approach is highly efficient, allowing for xenortide library development with every possible stereochemical combination, which is standard for SAR studies, following the mantra of medicinal and biological chemistry. Our retrosynthetic analysis for those compounds is shown in Scheme 1.

Results and discussion

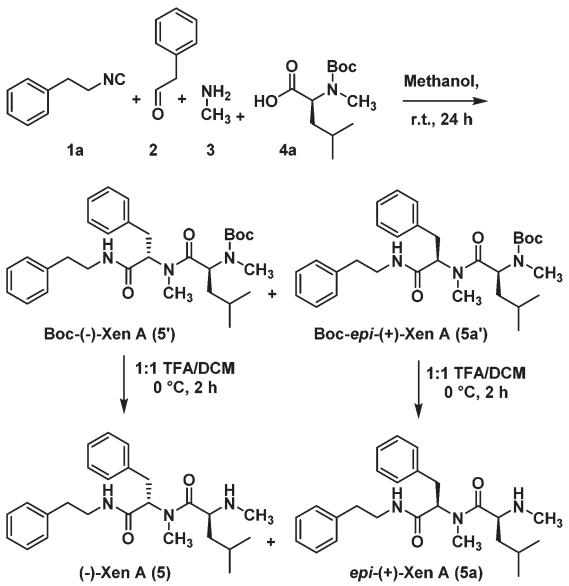
Chemistry

Synthetic efforts commenced with synthesizing phenylethyl isocyanide (**1a**[24]) and indole ethyl isocyanide (**1b**[25]) as required precursors in the Ugi 4-CR. These compounds were synthesized through common formylation[26] and dehydration[27,28] procedures. Formylation was achieved *via* refluxing the aryl ethylamine (**1a′** and **1b′**) with ethyl formate for 6 h. A solution of the related aryl ethyl formate (**1a″** and **1b″**) and Et_3N in anhydrous dichloromethane was treated with $POCl_3$ at −78 °C for 3 h to furnish the required isocyanides in nearly quantitative yields (Scheme 2).

Having precursors **1a** and **1b** in hand, we began our two-step synthetic protocol of (−)-Xen A (**5**) and *epi*-(+)-Xen A (**5a**) (Scheme 3). The reaction was designed to employ stoichiometric equivalents of phenylethyl isocyanide (**1a**), phenyl acetaldehyde (**2**), methylamine (**3**) (33% weight in ethanol) and Boc-*N*-methyl-L-leucine (**4a**) which readily dissolved in methanol as the solvent. The reaction was then stirred at room temperature for 24 h. The resultant mixture led to the formation of



Scheme 2 Formylation and dehydration for the synthesis of aryl isocyanides **1a** and **1b**.

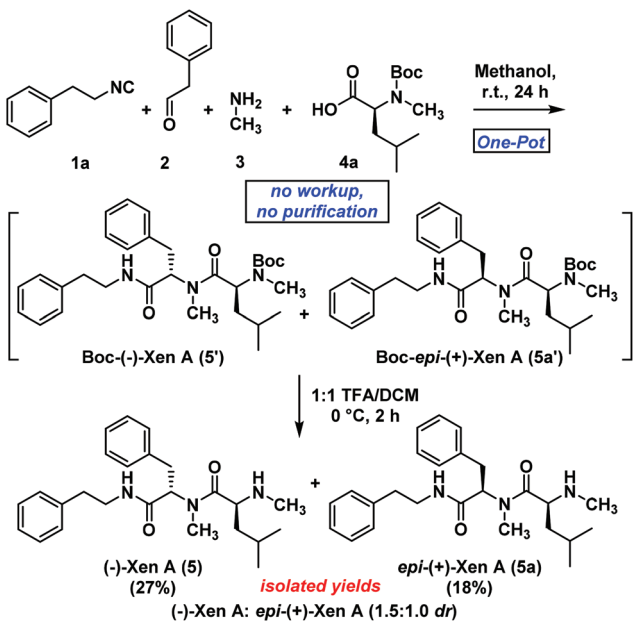


Scheme 3 Two-step synthesis of (−)-Xen A (**5**) and *epi*-(+)-Xen A (**5a**).

two diastereomers, which were readily visualized using TLC; R_f values of 0.5 and 0.8. However, due to the formation of rotamers during ^{1}H NMR analysis, we were unable to quantitate the actual diastereomeric ratios of the unpurified mixture of **5′** and **5a′**. We have performed NMR studies at high temperature in an attempt to clarify the rotameric issue, however, complete coalescence was not achieved. After careful separation of the Boc protected diastereomers using silica gel column chromatography with hexane and ethyl acetate as the eluents, ^{1}H NMR analysis was conducted for individual diastereomers **5′** and **5a′**. Unfortunately, rotamers were once again problematic and definitive NMR characterization continued to be elusive. Compounds **5′** and **5a′** were then subjected to Boc deprotection using 50% TFA in DCM at 0 °C for 2 h (Scheme 4). After TLC indicated complete Boc deprotection, isolation and purification of the individual (−)-Xen A (**5**) and *epi*-(+)-Xen A (**5a**) ensued using heptane and ethyl acetate as the solvents with normal phase silica gel chromatography. Pure compounds were then subjected to ^{1}H NMR spectral analysis and at this point we did not observe rotamers, which had plagued our ability to properly characterize other intermediates as previously stated. All of the NMR spectral analysis and the optical rotary values observed for compounds **5** and **5a** gave identical peak matching to previous literature reports.[13,14]

To by-pass the rotameric issue that we encountered with the initial two-step sequence and our problem quantifying diastereomeric ratios of intermediates using ^{1}H NMR analysis, we elected to alter our synthetic strategy and re-design our approach that would include a one pot, two-step synthetic process (Scheme 4).

To further support a more efficient and effective process to Xens A–D, the strategy was intended to avoid tedious workup and purifications of Boc-protected compounds such as **5′** and **5a′**.



Scheme 4 One-pot, two-step synthesis of **5** and **5a**.

In formalizing the one-pot, two-step synthetic process for (−)-Xen A (**5**) and *epi*-(+)-Xen A (**5a**) (Scheme 4), we decided to follow the exact route for the synthesis of the other Xen family of natural products. Therefore, in all cases, when TLC and mass spectral analysis indicated completion, methanol was evaporated under reduced pressure and without any further purification, the crude mixture was subjected to the Boc deprotection conditions which consisted of 50% TFA in DCM at 0 °C followed by stirring for 2 h.

The unpurified mixture of (+/−)-Xens A–D was then analyzed using ^{1}H NMR to determine diastereomeric ratios and then silica gel column chromatography with heptane and ethyl acetate as the solvent system was used to isolate our target molecules. Further NMR spectral analysis of the individually isolated pure compounds was conducted and peak shift values were observed matching exactly to those found in the literature.[13,14]

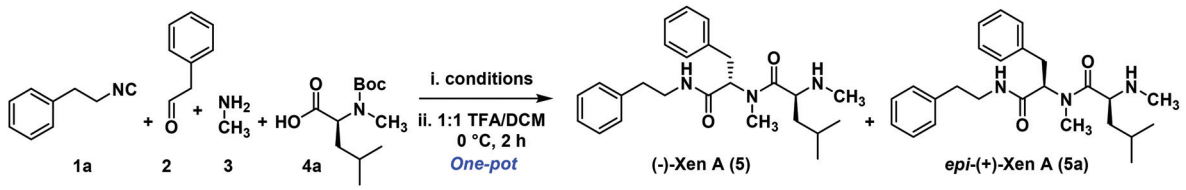
At this point in the study, we elected to conduct a detailed investigation on the effect of temperature on diastereoselectivity. Reactions were subjected to conditions at 0 °C, room temperature and 40 °C all the while maintaining consistency with a 24 h reaction time-frame using methanol as the solvent. From these studies, we observed that room temperature conditions gave the best diastereomeric ratios of 1.5 : 1 when compounds **1a**, **2**, **3**, **4a** were used (Table 1).

All the other natural Xens and their stereoisomers were obtained employing the conditions noted in entry 2; Table 1. Using the noted conditions enabled us to access a small library of Xen derivatives with isocyanides **1a** and **1b** along with L-Leu and L-Val. Diastereomeric ratios were again determined using 1H NMR and R_f values of each diastereomer in heptane/ethyl acetate (4 : 1) were noted as documented in Table 2.

In all cases, formation of diastereomers with (*S*) configuration at C_8 were favored over those with the (*R*) configuration (Scheme 5).[29–32] We assume that preferable formation of the (*S*)-diastereomer in all synthesized compounds can be explained in the detailed mechanism of (−)-Xen A (**5**) as a model in the Ugi 4-CR. In this mechanism, we propose that nucleophilic attack by the phenylethyl isocyanide (**1a**) occurs from the *Re*-face of the thermodynamically more stable (*E*)-imine, which forms first in the equilibrium sequence of events between **2** and **3** (**I**). We argue that this is made possible through hydrogen-bond donation of amino acids **4a–4d**, which block the *Si*-face of the (*E*)-imine and leads to the slightly preferred (*S*) configuration at C_8 (**II**). What follows next is attack on the nitrilium ion by the carboxylate leading to intermediate (**III**) which undergoes the Mumm rearrangement[30] in the rate determining step giving **5′**, which, when treated with TFA in DCM renders (−)-Xen A (**5**) as the major diastereomer after purification.

Under the conditions noted, all desired target products were obtained in fair to moderate yields. We were able to calculate the enantioselectivities for (−)-Xen A/B and *ent*-(+)-Xen A/B using specific optical rotation values of those compounds reported in literature[2] (Table 3). The excellent optical rotary

Table 1 Effect of temperature on the one-pot synthesis



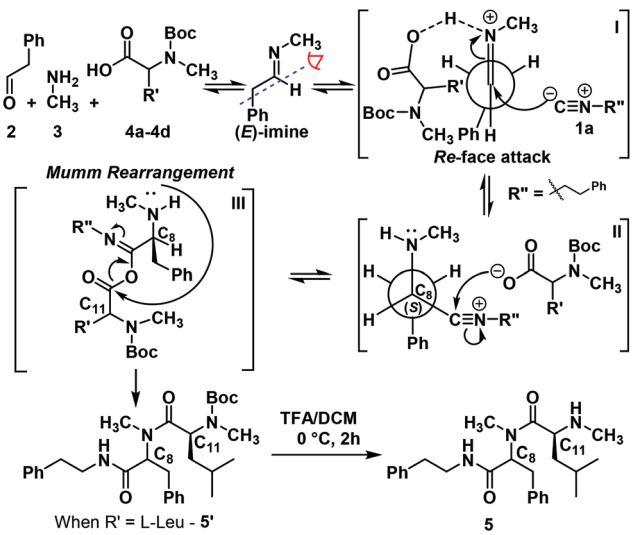
Entry	Conditions	Diastereomeric ratio [**5** (*S*,*S*) : **5a** (*S*,*R*)][a]	Yield[b] (%) **5** (*S*,*S*)	Yield[b] (%) **5a** (*S*,*R*)
1	MeOH, 0 °C, 24 h	1.3 : 1	15	<5
2	MeOH, r.t., 24 h	1.5 : 1	27	18
3	MeOH, 40 °C, 24 h	1.1 : 1	17	13

[a] Diastereomeric ratios determined using ^{1}H NMR analysis of the unpurified reaction mixture (T_1 = 3.5). [b] Compounds purified using silica gel chromatography with heptane and ethyl acetate as the solvents.

Table 2 A one-pot, two-step reaction sequence for (+/−) and *epi*-(+/−)-Xens

Entry	R	R′	Product[a]	Diastereomeric ratio[b]	R_f (cm)
1	Ph	L-Leu) – **4a**	(−)-Xen A (**5**)/*epi*-(+)-Xen A (**5a**)	1.5 : 1.0	0.5/0.8
2	3-Indole	L-Leu) – **4a**	(−)-Xen B (**6**)/*epi*-(+)-Xen B (**6a**)	1.5 : 1.0	0.3/0.6
3	Ph	L-Val) – **4b**	(−)-Xen C (**7**)/*epi*-(+)-Xen C (**7a**)	1.3 : 1.0	0.5/0.7
4	3-Indole	L-Val) – **4b**	(−)-Xen D (**8**)/*epi*-(+)-Xen D (**8a**)	1.3 : 1.0	0.4/0.7
5	Ph	D-Leu) – **4c**	*ent*-(+)-Xen A (**5b**)/*ent-epi*-(−)-Xen A (**5c**)	1.0 : 1.3	0.3/0.6
6	3-Indole	D-Leu) – **4c**	*ent*-(+)-Xen B (**6b**)/*ent-epi*-(−)-Xen B (**6c**)	1.0 : 1.4	0.5/0.8
7	Ph	D-Val) – **4d**	*ent*-(+)-Xen C (**7b**)/*ent-epi*-(−)-Xen C (**7c**)	1.0 : 1.1	0.5/0.8
8	3-Indole	D-Val) – **4d**	*ent*-(+)-Xen D (**8b**)/*ent-epi*-(−)-Xen D (**8c**)	1.0 : 1.5	0.4/0.6

[a] Compounds purified using silica gel chromatography with heptane and ethyl acetate as the solvents. [b] Diastereomeric ratios determined using ^{1}H NMR analysis of the unpurified reaction mixture.



Scheme 5 Plausible mechanism for diastereoselectivity in the Ugi 4-CR.

Table 3 Library of natural and unnatural Xens

Compound	Name	Yield[a] (%)	% ee[b]
5	(−)-Xen A	27	>98
5a	*epi*-(+)-Xen A	18	—
5b	*ent*-(+)-Xen A	15	>98
5c	*ent-epi*-(−)-Xen A	19	—
6	(−)-Xen B	28	>98
6a	*epi*-(+)-Xen B	19	—
6b	*ent*-(+)-Xen B	20	>98
6c	*ent-epi*-(−)-Xen B	28	—
7	(−)-Xen C	25	—
7a	*epi*-(+)-Xen C	19	—
7b	*ent*-(+)-Xen C	21	—
7c	*ent-epi*-(−)-Xen C	23	—
8	(−)-Xen D	20	—
8a	*epi*-(+)-Xen D	15	—
8b	*ent*-(+)-Xen D	17	—
8c	*ent-epi*-(−)-Xen D	25	—

[a] After silica gel column chromatography. [b] Enantiomeric excess (ee) was determined using specific optical rotation values reported for (−)-Xen A and B.[13]

values observed between our compounds and those published reveal that retention of chirality is maintained in the *N*-Boc protected amino acids (L/D-Leu) under our protocol. This further validates that under our noted reaction conditions no racemization at C_{11} occurred.

Biological studies

After completing the synthesis of Xen compounds noted in Table 3, we turned our attention to cytotoxicity analyses using five different cancer cell lines; colon carcinoma (HCT-116), human lung carcinoma (H460), human ovarian carcinoma

Table 4 Cytotoxic effects of naturally occurring Xens A–D and stereoisomers on the survival of cancer cell lines and normal cell lines

Compounds	Colon HCT-116[a]	Lung H460[a]	Ovarian OV-2008[a]	Breast MDA-MB-231[a]	Prostate DU-145[a]	Normal Cells CRL[a]	Normal Cells CHO[a]
(−)-Xen A	>100	>100	>100	95	>100	99	88
epi-(+)-Xen A	81	95	72	>100	94	>100	>100
ent-(+)-Xen A	86	97	65	88	98	>100	78
ent-epi-(−)-Xen A	46	78	58	60	67	>100	87
(−)-Xen B	19	24	25	21	25	86	54
epi-(+)-Xen B	48	67	59	55	67	63	78
ent-(+)-Xen B	48	>100	>100	41	67	>100	87
ent-epi-(−)-Xen B	25	58	60	20	42	87	59
(−)-Xen C	63	79	50	36	72	>100	92
epi-(+)-Xen C	62	75	72	59	85	>100	92
ent-(+)-Xen C	90	97	97	97	99	>100	>100
ent-epi-(−)-Xen C	>100	>100	>100	>100	97	>100	>100
(−)-Xen D	99	>100	>100	>100	>100	>100	>100
epi-(+)-Xen D	54	79	64	49	85	76	88
ent-(+)-Xen D	26	35	25	21	50	>100	84
ent-epi-(−)-Xen D	35	81	64	64	90	72	88
Cisplatin[b]	11	8	14	12	14	21	18

[a] The IC_{50} (μM) values are represented as mean ± SD of three independent experiments performed in triplicate. [b] Cisplatin was used as a positive control.

(OV-2008), human breast adenocarcinoma (MDA-MB-231), and human prostate adenocarcinoma (DU-145), as well as normal cell lines human epithelial colon (CRL-1459) and Chinese hamster ovarian cells (CHO) using the MTT assay.[33,34] The antiproliferative efficacy data are presented as IC_{50} values defined as the concentration of the compound that decreases cell proliferation at 50% (Table 4). The IC_{50} values were generated from at least three independent experiments. Cell survival was determined by MTT assay as described in materials and methods.

Cytotoxicity evaluation and the structure activity relationships study

The influence of chirality at C_8 and C_{11} on cytotoxicity of naturally occurring Xens A–D and all their stereoisomers (enantiomers and diastereomers) were investigated and some interesting results were observed from this focused library of compounds. Tryptamides Xen B and D stereoisomers were overall more active than the phenylethylamides Xen A and C stereoisomers. It was also previously reported that tryptamides (−)-Xen B and D, natural products were more active than natural phenylethylamides (−)-Xen A and C against sleeping sickness (*Trypanosoma brucei rhodesiense*) and malaria (*Plasmodium falciparum*) diseases.[14] These data together suggest that tryptamide analogues play a significant role in the enhancement of biological activity of the Xen family.

In addition, (−)-Xen B and *ent*-(+)-Xen D were found to be most active among all tested compounds. (−)-Xen B at 19–25 μM produced significant morphological changes against a total of five tested cancer cell lines, HCT-116, H460, OV-2008, MDA-MB-23, DU-145 (Fig. 1). Also, *ent*-(+)-Xen D had a 21–26 μM cytotoxicity, selective against three tested cancer cell lines that included HCT-116, OV-2008 and MDA-MB-23. Moreover, inspecting the cytotoxicity of active compounds ((−)-Xen B and *ent*-(+)-Xen D), in comparison to their related stereoisomers, revealed that although they have the same

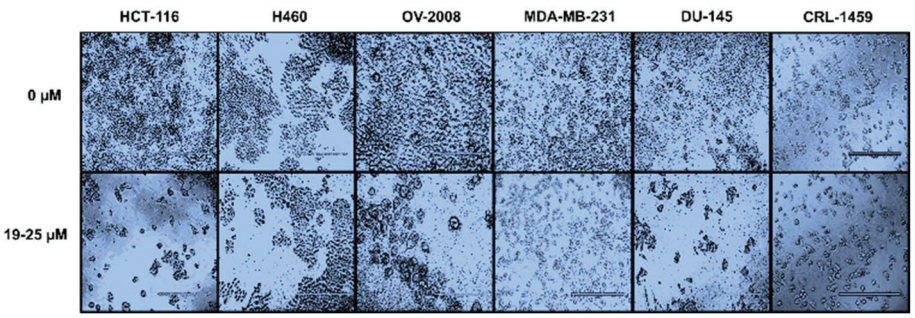


Fig. 1 Morphological analysis of the cytotoxic effects of (−)-Xen B at 0 and 19–25 μM on cancerous cell lines including human colon carcinoma (HCT-116), human lung carcinoma (H460), human ovarian carcinoma (OV-2008), human breast adenocarcinoma (MDA-MB-231), human prostate adenocarcinoma (DU-145) and normal human epithelial colon cell (CRL-1459). The cells were photographed at 20 °C, 72 h after incubation for each triplicate treatment.

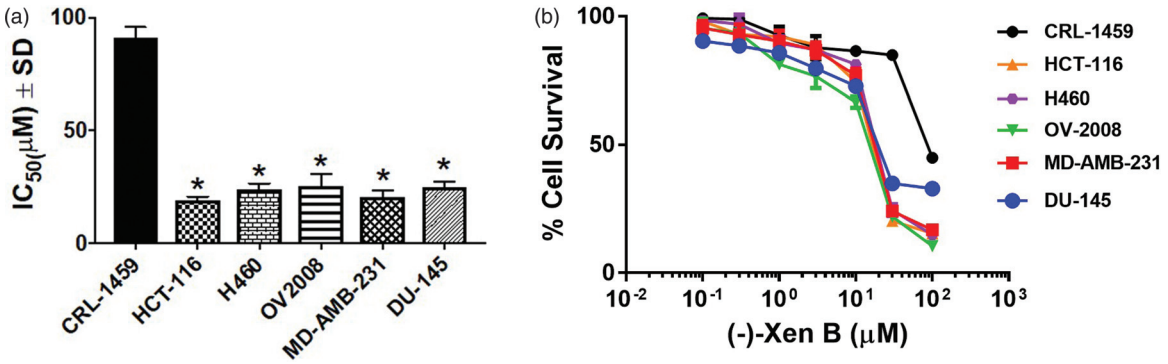


Fig. 2 (A) IC_{50} values and (B) cell survival curves of cancer cells compared to that of normal human epithelial colon cell (CRL-1459) when treated with (−)-Xen B determined by the MTT assay. IC_{50} values are represented as means ± SD of three independent experiments performed in triplicate.

chemical structures, they exhibit distinct differences in anticancer activities. For example, natural (−)-Xen B in comparison to its enantiomer, *ent*-(+)-Xen B, revealed markedly more cytotoxic activity (IC_{50} = 19–25 μM) than its enantiomer (IC_{50} = 41–100 μM) in all evaluated cancer lines. Similarly, *ent*-(+)-Xen D exhibited significantly higher cytotoxic activity than its enantiomer (−)-Xen D, with IC_{50} values of 21–50 μM and >100 μM, respectively. Furthermore, in this study, the potency of the most active compounds was considerably greater for cancer cells when compared to the normal cells, meaning that no toxicity was recorded toward normal cell lines CRL-1459 and CHO. (These compounds were noted to be considerably safe when tested on the normal cell lines.) (Fig. 2). Cisplatin, a well-known anticancer agent, was used as a positive control in our experiments. We observed that although, cisplatin killed the cancer cells at a concentration range of 8–14 μM, they were less selective compared to Xens on normal cells (Table 4).

The cytotoxicity effects of active (−)-Xen B and *ent*-(+)-Xen D on various cell lines (cancerous and non-cancerous cells)

Based on our data, for (−)-Xen B and the cohort of stereoisomers, the chiral properties of C_8 containing the (*S*) stereoconfiguration is considered a critical factor influencing compound potency. As summarized in Table 4, (−)-Xen B (highlighted in yellow) with (*S*) configuration at C_8, was determined to have excellent cytotoxic activities (IC_{50} = 19–25 μM) against five cancer cell lines, and was determined to be approximately 2-fold more potent than its corresponding diastereomer, *epi*-(+)-Xen B with the (*R*) configuration at C_8. Coincident results were observed for *ent-epi*-(−)-Xen B and *ent*-(+)-Xen B, with IC_{50} values of 20–60 μM and 41 → 100 μM, respectively. These results strongly suggest that the chirality at C_8 plays a substantial role in cytotoxic activity of (−)-Xen B stereoisomers.

Moreover, *ent*-(+)-Xen D (highlighted in yellow) was selectively active against three cancer cell lines, including HCT-116, MDA-MB-231 and OV-2008. In those three cancer cell lines, *ent*-(+)-Xen D with (*R*) configuration at C_{11} revealed better cytotoxic effects than its diastereomer, *epi*-(+)-Xen D, with respective IC_{50} values of 21–35 μM and 49 → 79 μM. Similar results were observed for *ent-epi*-(−)-Xen D and (−)-Xen D, proving that the stereochemistry of C_{11} in (−)-Xen D plays an important role in cytotoxic activity.

Conclusion

In conclusion, a concise synthesis of natural Xens A–D and all subsequent stereoisomers has been achieved in a one-pot, two-step Ugi 4-CR synthetic strategy. In this reaction, chiral *N*-Boc protected amino acids (L-Leu/Val and D-Leu/Val), aryl isocyanides, phenyl acetaldehyde and methylamine were utilized as starting materials. All reactions were carried out efficiently and simple chromatographic purification led to enantiomeric excesses (>90% ee) in reasonable yields. Based on our knowledge of the literature, the anticancer activities of sixteen Xen family of natural products and all their subsequent stereoisomers are reported here for the very first time. When dealing with biological interactions especially for medicinal applications, comparing the activity of stereoisomers is crucial, as biological targets are chiral. The stereo-SAR study demonstrated that only two compounds, among sixteen synthesized, were very active in the MTT assay. Natural (−)-Xen B revealed high cytotoxic activity against five tested cancer cell lines and *ent*-(+)-Xen D indicated decent cytotoxicity/selectivity against three cancer cell lines, described in Table 4. Furthermore, SAR results indicated that the stereochemistry at C_8 and C_{11} in (−)-Xen B and *ent*-(+)-Xen D, respectively, plays an important role in cytotoxic activities. Moreover, it should be noted that most of the compounds, especially those that are considerably active, were significantly less toxic toward normal cells, when compared to cancer cells, suggesting that the Xen analogues show selective toxicity. Cisplatin, a well-known anticancer agent, was used as a positive control in our experiments and Xen were more selective compared to cisplatin on normal cells. Finally, the results suggest that the stereochemistry of Xens could possibly play a vital role in the enhancement of bioactivity. Therefore, finding the target binding site should reveal more information about the connection between chiral properties and bioactivity of Xens in the future.[35,36]

Experimental

Methods and materials

Chemistry. All reagents and solvents purchased were used without further purification unless otherwise stated. Reaction progress was monitored using thin layer chromatography (TLC). TLC was visualized using UV. Column chromatography was performed using Whatman Purasil 60 A (230–400 mesh ASTM) silica gel; yields refer to chromatographically and spectroscopically pure compounds. ^{1}H and ^{13}C NMR were recorded using a Bruker Avance III 600 spectrometer at 22 °C (default) unless otherwise noted. The residual solvent signal in CD_3OD was referenced to 3.31 and 49.0 ppm in proton and carbon spectra respectively. Signal patterns are indicated as s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet; dd: doublet of doublets; br: broad and coupling constants are reported in hertz (Hz). Low resolution mass spectra (LRMS) were acquired on an Esquire-LC electrospray ionization (ESI) mass spectrometer. High resolution mass spectra (HRMS) were obtained with a Bruker Maxis 4G mass spectrometer.

General procedure. To 0.27 mL (1.66 mmol) of methylamine (33% in ethanol) (**3**) in 15 mL methanol, was added 0.18 mL (1.66 mmol) phenyl acetaldehyde (**2**), and the reaction was allowed to stir for 5 minutes at room temperature. Then, 1.66 mmol of corresponding carboxylic acid **4a–b**, and 1.66 mmol of isocyanide **1a–b** were added sequentially and the reaction was continuously stirred until no noticeable starting reagents were visualized on TLC. Upon completion of the reaction, methanol was evaporated under reduced pressure. After obtaining mass of the unpurified Boc-protected product, the material was dissolved in CH_2Cl_2 (1 mL) and trifluoroacetic acid (1 mL) was added as the reaction mixture was allowed to chill in an ice-bath for 2 h. The reaction was monitored for completion by TLC and the contents were then concentrated under reduced pressure. The crude compound was then subjected to flash column chromatography (EtOAc/hexanes) to yield pure compounds 5–8(a–c).

*(S)-N,4-Dimethyl-2-(methylamino)-N-((S)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)pentanamide (**5**).* The compound was obtained as a white solid; yield: (185.01 mg, 27%); $[\alpha]_D^{20}$ = −57° (*c* 0.2, MeOH); lit.[13] = −54° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 4.3/1; major rotamer noted: δ 7.29–7.24 (m, 6H, aryl), 7.21–7.17 (m, 4H, aryl), 5.42–5.39 (dd, J_1 = 6.0 Hz, J_2 = 12.0 Hz, 1H, CH), 3.43–3.41 (t, J = 7.2 Hz, 2H, CH_2), 3.38–3.36 (m, 1H, CH), 3.18–3.03 (dd, J_1 = 5.4 Hz, J_2 = 14.4 Hz, 1H, CH), 3.11–3.00 (m, 1H, CH), 2.97 (s, 3H, CH_3–N), 2.78–2.76 (t, J = 7.2 Hz, 2H, CH_2), 1.74 (s, 3H, CH_3–NH), 1.30–1.25 (m, 2H, CH_2), 1.15–1.14 (m, 1H, CH), 0.92–0.89 (m, 6H, $2CH_3$); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.84, 170.75, 138.97, 137.13, 128.87, 128.74, 128.51, 128.14, 126.34, 126.01, 58.01, 57.57, 42.04, 40.46, 35.04, 34.22, 32.72, 30.70, 24.24, 22.49, 20.97 ppm; **HRMS** (TOF-ESI): $[M + H]^+$calculated for $C_{25}H_{36}N_3O_2$: 410.2808; found: 410.2807.

*(S)-N,4-Dimethyl-2-(methylamino)-N-((R)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)pentanamide (**5a**).* The compound was obtained as a white solid; yield: (127.59 mg, 18%); $[\alpha]_D^{20}$ = +43° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD); δ 7.28–7.20 (m, 10H, aryl), 5.54–5.51 (dd, J_1 = 6.0 Hz, J_2 = 12.0 Hz, 1H, aryl), 3.77–3.75 (m, 1H, CH), 3.48–3.44 (m, 2H, CH_2), 3.31–3.25 (dd, J_1 = 6.0 Hz, J_2 = 12.0 Hz, 1H, CH_2), 3.00–2.92 (m, 4H, CH, CH_3–N), 2.82–2.80 (t, J = 6.0 Hz, 2H, CH_2), 2.37 (s, 3H, CH_3–NH), 1.10–1.07 (m, 2H, CH_2), 0.89–0.85 (m, 1H, CH), 0.74–0.73 (d, J = 6.0 Hz, 3H, CH_3), 0.69–0.68 (d, J = 6.0 Hz, 3H, CH_3); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 174.85, 170.78, 138.98, 136.87, 128.49, 128.45, 128.20, 128.13, 126.55, 126.02, 57.45, 57.21, 41.03, 40.56, 35.01, 34.35, 32.61, 30.46, 23.83, 21.92, 21.33 ppm; **HRMS** (TOF-ESI): $[M + H]^+$calculated for $C_{25}H_{36}N_3O_2$: 410.2808; found: 410.2820.

*(R)-N,4-Dimethyl-2-(methylamino)-N-((R)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)pentanamide (**5b**).* The compound was obtained as a white solid; yield: (102.30 mg, 15%); $[\alpha]_D^{20}$ = +57° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 3.5/1; major rotamer noted: δ 7.28–7.21 (m, 6H, aryl), 7.19–7.16 (m, 4H, aryl), 5.43–5.40 (dd, J_1 = 6.0 Hz, J_2 = 12.0 Hz, 1H, CH), 3.44–3.36 (t, J = 7.2 Hz, 2H, CH_2), 3.35–3.33 (m, 1H), 3.20–3.17 (dd, J_1 = 5.2 Hz, J_2 = 14.3 Hz, 1H), 3.01–2.96 (m, 4H, CH, CH_3–N), 2.78–2.76 (t, J = 7.2 Hz, 2H, CH_2), 1.73 (s, 3H, CH_3–NH), 1.29–1.24 (m, 2H, CH_2), 1.14–1.13 (m, 1H, CH), 0.91–0.88 (m, 6H, $2CH_3$); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 176.00, 170.75, 139.04, 137.48, 128.88, 128.74, 128.52, 126.60, 126.34, 126.07, 58.01, 57.99, 56.22, 40.47, 35.09, 34.24, 32.80, 30.71, 24.24, 21.95, 21.01 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{25}H_{36}N_3O_2$: 410.2808; found: 410.2823.

*(R)-N,4-Dimethyl-2-(methylamino)-N-((S)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)pentanamide (**5c**).* The compound was obtained as a white solid; yield: (133.02 mg, 19%); $[\alpha]_D^{20}$ = −43° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD); δ = 7.29–7.17 (m, 10H, aryl), 5.55–5.52 (dd, J_1 = 5.3 Hz, J_2 = 12.0 Hz, 1H, CH), 3.46–3.40 (m, 2H, CH_2), 3.39–3.38 (m, 1H, CH), 3.25–3.22 (dd, J_1 = 5.3 Hz, J_2 = 12.0 Hz, 1H, CH), 2.98–2.93 (m, 4H, CH, CH_3–N), 2.81–2.78 (m, 2H, CH_2), 2.20 (s, 3H, CH_3–NH), 0.99–0.95 (m, 1H, CH), 0.83–0.78 (m, 1H, CH), 0.77–0.74 (m, 1H, CH), 0.75 (d, J = 6.0 Hz, 3H, CH_3), 0.66 (d, J = 6.0 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD): δ 176.43, 170.89, 138.99, 136.95, 128.49, 128.47, 128.21, 128.16, 126.47, 126.01, 57.36, 57.19, 41.74, 40.53, 35.03, 34.32, 33.11, 30.41, 23.91, 21.99, 21.49 ppm; **HRMS** (TOF-ESI): $[M + H]^+$calculated for $C_{25}H_{36}N_3O_2$: 410.2808; found: 410.2816.

*(S)-N-((S)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,4-dimethyl-2-(methylamino)pentanamide (**6**).* The compound was obtained as a yellowish solid; yield: (215.77 mg, 28%); $[\alpha]_D^{20}$ = −56° (*c* 0.3, MeOH); lit.[13] −58° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 2.8/1; major rotamer noted: δ 7.56–7.55 (d, J = 6.0 Hz, 1H, aryl), 7.33–7.32 (d, J = 6.0 Hz, 1H, aryl), 7.26–7.23 (m, 4H, aryl), 7.19–7.16 (m, 1H, aryl), 7.08–7.06 (t, J = 7.2 Hz, 1H, aryl), 7.04–6.99 (m, 2H, aryl), 5.40–5.39 (dd, J_1 = 6.0, J_2 = 12.0 Hz, 1H, CH), 3.57–3.50 (m, 2H, CH_2), 3.36–3.35 (m, 1H, CH), 3.17–3.15 (dd, J_1 = 5.4 Hz, J_2 =

14.4 Hz, 1H, CH), 3.01–2.95 (m, 6H, CH, CH_2, CH_3–N), 1.73 (s, 3H, CH_3–NH), 1.37–1.21 (m, 2H, CH_2), 1.07–1.06 (m, 1H, CH), 0.98–0.84 (m, 6H, $2CH_3$) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.72, 170.68, 136.78, 128.74, 128.43, 128.13, 127.39, 126.32, 121.05, 120.96, 118.23, 117.88, 111.61, 110.89, 58.17, 57.56, 39.84, 39.77, 32.67, 30.77, 30.64, 24.77, 24.26, 22.41, 20.98 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{27}H_{37}N_4O_2$: 449.2917 found 449.2933.

*(S)-N-((R)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,4-dimethyl-2-(methylamino)pentanamide (**6a**).* The compound was obtained as a yellowish solid; yield: (142.97 mg, 19%); $[\alpha]_D^{20}$ = +26° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 2.68/1; major rotamer noted: δ 7.57–7.56 (d, J = 7.8 Hz, 1H, aryl), 7.33–7.31 (d, J = 7.8 Hz, 1H, aryl), 7.25–7.20 (m, 4H, aryl), 7.19–7.17 (t, J = 7.2 Hz, 1H, aryl), 7.09–7.06 (m, 2H, aryl), 7.02–7.01 (t, J = 7.8 Hz, 1H, aryl), 5.54–5.51 (dd, J_1 = 5.4 Hz, J_2 = 11.4 Hz, 1H, CH), 3.55–3.52 (m, 2H, CH_2), 3.40–3.37 (m, 1H, CH), 3.17–3.15 (dd, J_1 = 5.4, J_2 = 15.0 Hz, 1H, CH), 2.99–2.94 (m, 3H, CH, CH_2), 2.88 (s, 3H, CH_3–N), 2.21 (s, 3H, CH_3–NH), 0.96–0.95 (m, 2H, CH_2), 0.84–0.79 (m, 1H, CH_2), 0.80–0.69 (d, J = 6.0 Hz, 3H, CH_3), 0.65–0.64 (d, J = 6.0 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 176.17, 170.78, 136.95, 136.77, 128.65, 128.16, 127.42, 126.46, 122.17, 120.96, 118.23, 117.91, 111.58, 110.86, 57.45, 57.19, 41.58, 39.87, 34.13, 30.34, 30.25, 24.63, 24.34, 22.47, 21.42 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{27}H_{37}N_4O_2$: 449.2917 found 449.2931.

*(R)-N-((R)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,4-dimethyl-2-(methylamino)pentanamide (**6b**).* The compound was obtained as a yellowish solid; yield: (151.63 mg, 20%); $[\alpha]_D^{20}$ = +56° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 2.7/1; major rotamer noted: δ 7.56–7.55 (m, 1H, aryl), 7.33–7.17 (m, 4H, aryl), 7.09–7.00 (m, 5H, aryl), 5.41–5.39 (dd, J_1 = 5.6, J_2 = 11.0 Hz, 1H, CH), 3.52–3.50 (m, 2H, CH_2), 3.34–3.32 (m, 1H, CH), 3.19–3.15 (dd, J_1 = 5.6 Hz, J_2 = 14.4 Hz, 1H, CH), 3.00–2.91 (m, 6H, CH, CH_2, CH_3–N), 1.69 (s, 3H, CH_3–NH), 1.29–1.20 (m, 2H), 1.07–1.06 (m, 1H, CH), 0.86–0.84 (m, 6H, $2CH_3$) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 176.03, 170.71, 137.19, 128.85, 128.42, 128.12, 127.38, 126.31, 122.07, 120.95, 118.23, 117.87, 110.96, 110.89, 61.43, 57.57, 43.08, 39.83, 34.08, 32.74, 30.63, 24.77, 24.27, 22.42, 20.99 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{27}H_{37}N_4O_2$: 449.2917 found 449.2914.

*(R)-N-((S)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,4-dimethyl-2-(methylamino)pentanamide (**6c**).* The compound was obtained as a yellowish solid; yield: (212.30 mg, 28%); $[\alpha]_D^{20}$ = −26° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 6.0/1; major rotamer noted: δ = 7.57–7.56 (d, J = 7.8 Hz, 1H, aryl), 7.33–7.31 (d, J = 7.8 Hz, 1H, aryl), 7.25–7.17 (m, 5H, aryl), 7.10–7.07 (m, 2H, aryl), 7.02–7.01 (t, J = 7.8 Hz, 1H, aryl), 5.54–5.52 (dd, J_1 = 4.32 Hz, J_2 = 11.0 Hz, 1H, CH), 3.54–3.36 (m, 2H, CH_2), 3.35–3.31 (m, 1H), 3.23–3.20 (dd, J_1 = 5.4, J_2 = 15.0 Hz, 1H, CH), 2.99–2.94 (m, 3H, CH, CH_2), 2.88 (s, 3H, CH_3–N), 2.21 (s, 3H, CH_3–NH), 0.96–0.93 (m, 2H, CH_2), 0.84–0.79 (m, 1H, CH), 0.80–0.69 (d, J = 6.0 Hz, 3H, CH_3), 0.65–0.64 (d, J = 6.0 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 176.40, 170.82, 136.99, 136.78, 128.66, 128.14, 127.42, 126.44, 122.17, 120.95, 118.22, 117.90, 111.58, 110.85, 57.40, 57.15, 41.72, 39.84, 34.14, 32.98, 30.29, 24.63, 23.89, 21.96, 21.46; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{27}H_{37}N_4O_2$: 449.2917 found 449.2931.

*(S)-N,3-Dimethyl-2-(methylamino)-N-((S)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)butanamide (**7**).* The compound was obtained as a white solid; yield: (166.99 mg, 25%); $[\alpha]_D^{20}$ = −54° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 7.70/1; major rotamer noted: δ 7.29–7.27 (m, 6H, aryl), 7.22–7.7.19 (m, 4H, aryl), 5.51–5.49 (dd, J_1 = 5.4 Hz, J_2 = 11.4 Hz, 1H, CH), 3.50–3.44 (t, J = 7.2 Hz, 2H, CH_2), 3.22–2.99 (m, 6H, CH, CH_2, CH_3–N), 2.07–2.04 (m, 1H, CH), 1.97 (s, 3H, CH_3–NH), 1.04–1.03 (d, J = 6.6 Hz, 3H, CH_3), 0.98–0.97 (d, J = 6.6 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 170.13, 167.65, 138.92, 136.91, 128.46, 128.23, 128.18, 128.13, 126.54, 126.03, 63.45, 58.06, 40.44, 34.98, 34.97, 34.05, 31.17, 29.81, 17.67, 16.10 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{24}H_{34}N_3O_2$: 396.2651; found: 396.2645.

*(S)-N,3-Dimethyl-2-(methylamino)-N-((R)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)butanamide (**7a**).* The compound was obtained as a white solid; yield: (129.45 mg, 19%); $[\alpha]_D^{20}$ = +29° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 2.96/1; major rotamer noted: δ 7.29–7.26 (m, 6H, aryl), 7.22–7.20 (m, 4H, aryl), 5.55–5.49 (dd, J_1 = 5.4 Hz, J_2 = 12.0 Hz, 1H, CH), 3.50–3.46 (m, 2H, CH_2), 3.27–3.23 (m, 2H, CH_2), 3.02–2.98 (m, 4H, CH, CH_3–N), 2.83–2.81 (t, J = 7.2 Hz, 2H, CH_2), 2.48 (s, 3H, CH_3–NH), 1.66–1.59 (m, 1H, CH), 0.75–0.74 (d, J = 6.6 Hz, 3H, CH_3), 0.53–0.52 (d, J = 6.6 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 170.53, 170.19, 138.98, 136.79, 128.52, 128.50, 128.34, 128.14, 126.60, 126.04, 63.97, 57.91, 40.59, 34.99, 34.28, 30.76, 29.51, 17.47, 15.66, 13.04 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{24}H_{34}N_3O_2$: 396.2651; found: 396.2640.

*(R)-N,3-Dimethyl-2-(methylamino)-N-((R)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)butanamide (**7b**).* The compound was obtained as a white solid; yield: (142.68 mg, 21%); $[\alpha]_D^{20}$ = +54° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 4.2/1; major rotamer noted: δ 7.29–7.23 (m, 6H, aryl), 7.20–7.17 (m, 4H, aryl), 5.51 (d.d, J_1 = 5.4, J_2 = 10.8 Hz, 1H, CH), 3.44 (t, J = 6.6 Hz, 2H, CH_2), 3.22–3.21 (m, 2H, 2CH), 3.00–2.96 (m, 4H, CH, CH_3–N), 2.78–2.75 (m, 2H, CH_2), 1.71–1.66 (m, 4H, CH, CH_3–NH), 0.92–0.88 (m, 6H, $2CH_3$); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.55, 170.46, 138.98, 137.03, 128.97, 128.63, 128.48, 128.19, 128.13, 126.38, 64.42, 57.52, 40.51, 35.03, 34.31, 33.65, 30.50, 30.28, 18.37, 16.24; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{24}H_{34}N_3O_2$: 396.2651; found: 396.2644.

*(R)-N,3-Dimethyl-2-(methylamino)-N-((S)-1-oxo-1-(phenethylamino)-3-phenylpropan-2-yl)butanamide (**7c**).* The compound

was obtained as a white solid; yield: (156.95 mg, 23%); $[\alpha]_D^{20}$ = −29° (*c* 0.2, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 6.3/1; major rotamer noted: δ 7.29–7.23 (m, 7H, aryl), 7.20–7.17 (m, 3H, aryl), 5.51 (d.d, J_1 = 5.4, J_2 = 10.8 Hz. 1H, CH), 3.44 (t, J = 6.6 Hz, 2H, CH_2), 3.22–3.21 (m, 2H, CH_2), 2.99–2.93 (m, 4H, CH, CH_3–N), 2.83–2.77 (m, 2H, CH_2), 2.17 (s, 3H, CH_3–NH), 1.40–1.36 (1H, m, CH), 0.63 (d, J = 6.6 Hz, 3H, CH_3), 0.53 (d, J = 6.6 Hz, 3H, CH_3); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.55, 170.46, 138.98, 137.03, 128.97, 128.63, 128.48, 128.19, 128.13, 126.38, 64.42, 57.52, 40.51, 35.03, 34.31, 33.65, 30.50, 30.28, 18.37, 16.24; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{24}H_{34}N_3O_2$: 396.2651; found: 396.2644.

*(S)-N-((S)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,3-dimethyl-2-(methylamino)butanamide (**8**).* The compound was obtained as a yellowish solid; yield: (142.02 mg, 20%); $[\alpha]_D^{20}$ = −58° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 3.8/1; major rotamer noted: δ 7.55–7.54 (d, J = 7.8 Hz, 1H, aryl), 7.33–7.31 (d, J = 7.8 Hz, 1H, aryl), 7.23–7.18 (m, 4H, aryl), 7.16–7.15 (m, 1H, aryl), 7.09–7.08 (t, J = 7.2 Hz, 1H, aryl), 7.04 (s, 1H, aryl), 6.99 (t, J = 7.2 Hz, 1H, aryl), 5.47 (dd, J_1 = 6.0 Hz, J_2 = 10.8 Hz, 1H, CH), 3.52–3.50 (m, 2H, CH_2), 3.18–3.14 (m, 1H, CH), 3.10–3.08 (d, J = 6.0 Hz, 1H, CH), 2.99–2.91 (m, 6H, CH, CH_2, CH_3–N), 1.68 (s, 3H, CH_3–NH), 1.60–1.57 (m, 1H, CH), 0.84–0.83 (d, J = 6.6 Hz, 3H, CH_3), 0.81 (d, J = 6.6 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.35, 170.66, 137.15, 136.80, 128.72, 128.35, 128.11, 126.30, 122.07, 120.95, 118.22, 117.87, 111.50, 110.86, 64.47, 57.65, 39.64, 34.03, 33.03, 30.93, 30.57, 24.79, 18.55, 16.40 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{26}H_{35}N_4O_2$: 435.2760; found: 435.2754.

*(S)-N-((R)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,3-dimethyl-2-(methylamino)butanamide (**8a**).* The compound was obtained as a white solid; yield: (109.67 mg, 15%); $[\alpha]_D^{20}$ = +28° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 6.6/1; major rotamer noted: δ 7.56–7.55 (d, J = 7.8 Hz, 1H, aryl), 7.33–7.32 (d, J = 7.8, 1H, aryl), 7.24–7.21 (m, 4H, aryl), 7.17–7.7.15 (m, 1H, aryl), 7.09–7.08 (t, J = 7.2 Hz, 1H, aryl), 7.05 (s, 1H, aryl), 7.01–6.99 (t, J = 7.2 Hz, 1H, aryl), 5.52–5.49 (dd, J_1 = 5.4 Hz, J_2 = 11.4 Hz, 1H, CH), 3.53–3.51 (t, J = 6.6 Hz, 2H, CH_2), 3.22–3.18 (m, 2H, CH_2), 2.96–2.90 (m, 6H, CH, CH_2, CH_3–N), 2.08 (s, 3H, CH_3–NH), 1.36–1.29 (m, 1H, CH), 0.62–0.60 (d, J = 6.6 Hz, 3H, CH_3), 0.51–0.50 (d, J = 6.6 Hz, 3H, CH_3) ppm; **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.36, 170.85, 137.06, 136.78, 128.62, 128.17, 127.39, 126.36, 122.15, 120.96, 118.23, 117.89, 110.43, 110.86, 64.38, 57.60, 39.79, 33.38, 33.49, 30.49, 30.25, 24.66, 18.32, 16.21 ppm; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{26}H_{35}N_4O_2$: 435.2760 found 435.2775.

*(R)-N-((R)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,3-dimethyl-2-(methylamino)butanamide (**8b**).* The compound was obtained as a yellowish solid; yield: (121.13 mg, 17%); $[\alpha]_D^{20}$ = +58° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 3.2/1; major rotamer noted: δ 7.55 (d, J = 7.8 Hz, 1H, aryl), 7.32 (d, J = 7.8 Hz, 1H, aryl), 7.24–7.22 (m, 1H, aryl), 7.18–7.15 (m, 1H, aryl), 7.08 (t, J = 7.8 Hz, 1H, aryl), 7.04–7.00 (m, 1H, aryl), 5.48 (d.d, J_1 = 5.4, J_2 = 11.4 Hz, 1H, CH), 3.53–3.50 (m, 2H, CH_2), 3.17–3.12 (m, 2H, CH_2), 2.99–2.92 (m, 6H, CH, CH_2, CH_3–N), 1.70 (s, 3H, CH_3–NH), 1.62–1.58 (m, 1H, CH), 0.90 (d, J = 7.2 Hz, 3H, CH_3), 0.82 (d, J = 7.2 Hz, 3H, CH_3); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 171.43, 170.64, 137.15, 136.80, 128.73, 128.11, 127.37, 126.30, 121.05, 120.95, 118.22, 117.87, 111.51, 110.86, 64.45, 57.66, 39.64, 34.09, 32.99, 30.89, 30.58, 24.79, 18.57, 16.67; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{26}H_{35}N_4O_2$: 435.2760; found: 435.2755.

*(R)-N-((S)-1-((2-(1H-Indol-3-yl)ethyl)amino)-1-oxo-3-phenylpropan-2-yl)-N,3-dimethyl-2-(methylamino)butanamide (**8c**).* The compound was obtained as a white solid; yield: (182.30 mg, 25%); $[\alpha]_D^{20}$ = −28° (*c* 0.3, MeOH); **^{1}H NMR** (600 MHz, CD_3OD) showed the presence of two rotamers in a ratio of 5.9/1; major rotamer noted: δ 7.56–7.54 (d, J = 7.8 Hz, 1H, aryl), 7.33–7.31 (d, J = 7.8 Hz, 1H, aryl), 7.22–7.20 (m, 4H, aryl), 7.17–7.15 (m, 1H, aryl), 7.08–7.07 (t, J = 7.8 Hz, 1H, aryl), 7.05 (s, 1H, aryl), 7.02–7.01 (m, 1H, aryl), 5.52–5.49 (d.d, J_1 = 6.0, J_2 = 12.0 Hz, 1H, CH), 3.53–3.51 (t, J = 7.2 Hz, 2H, CH_2), 3.21–3.18 (d.d, J_1 = 3.6, J_2 = 15.0 Hz, 1H, CH), 3.15–3.14 (d, J = 5.4, 1H, CH), 2.96–2.93 (m, 3H, CH, CH_2), 2.88 (s, 3H, CH_3–N), 2.07 (s, 3H, CH_3–NH), 1.34–1.33 (m, 1H, CH), 0.60 (d, J = 6.6 Hz, 3H, CH_3), 0.50 (d, J = 6.6 Hz, 3H, CH_3); **^{13}C NMR** (150 MHz, CD_3OD) major rotamer noted: δ 175.59, 170.86, 137.79, 128.63, 128.52, 128.17, 127.40, 126.35, 122.16, 120.97, 118.25, 117.91, 111.58, 110.88, 64.41, 57.60, 39.80, 34.13, 33.55, 30.44, 30.29, 24.68, 18.37, 16.25; **HRMS** (TOF-ESI): $[M + H]^+$ calculated for $C_{26}H_{35}N_4O_2$: 435.2760; found: 435.2726.

Biology

Materials. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Calbiochem EMD Millipore (Billerica, MA, USA). Dulbecco's modification of Eagle's medium (DMEM) and 0.25% trypsin + 2.20 mM ethylenediaminetetraacetic acid (EDTA), phosphate buffered saline (PBS without calcium or magnesium), and DMEM (phenol red – free) were purchased from Mediatech, Inc. (Corning subsidiaries, Manassas, VA, USA). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Flowery Branch, GA, USA). Penicillin/streptomycin was purchased from Lonza, Inc. (Allendale NJ, USA). Dimethyl sulfoxide (DMSO) was purchased from VWR Analytical (Radnor, PA, USA).

Cell lines and culture

Cancer cells, including colon (HCT-116), lung (H460), ovarian (OV-2008), breast (MDA-MB-231), prostate (DU-145), and normal cell lines including human epithelial colon (CRL-1459) and Chinese hamster ovary (CHO) were used in this study. All the cell lines were grown as adherent monolayers in flasks with DMEM cultured media supplemented with 10% FBS and 1% streptomycin/penicillin in a humidified incubator with 5% CO_2 at 37 °C.

Cell cytotoxicity assays by MTT

To determine the cytotoxicity of the natural Xens A–D and all stereoisomers thereof, a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT colorimetric assay was used, as previously described.[37] Briefly, after harvesting the cells with 0.25% trypsin and 2.21 mM EDTA, 1×, cancer cell lines were re-suspended in DMEM. Cells were seeded evenly (180 μl per well) into 96 well-plates in triplicate at 5000 cells per well and the plates were returned to the incubator, allowing cells to attach to the wells, for up to 24 h. On the second day of the experiment, various concentrations of each compound ranging from 0.10 to 100.0 μM (20.0 μL per well) were added to the wells. After 72 h of incubation, 20.0 μL of the MTT solution (4.0 mg mL^{-1}) was added to each well. The plates were further incubated for 4 h to allow the viable cells to bio transform the yellow-colored MTT into dark-blue formazan crystals. The medium was discarded and 100.0 μL of DMSO was used to dissolve the formazan crystals. The absorbance was measured at 570 nm using the SpectraMax® iD3 H1 Multi-Mode Micro Reader from Molecular Devices (Sunnyvale, CA, USA). The IC_{50} values were calculated from the cell survival percentages. Similarly, the cytotoxicity of the test compounds was compared to normal cell lines CHO and CRL-1459.

Cell morphological analysis

Cell images were taken using Evos® FL cell imaging system microscope from Thermo-Fisher Scientific (Waltham, MA) with a fluorescent lamp and digital camera. Morphological changes were observed in cancer and normal cell lines 72 h after incubation with different concentrations of Xen compounds.

Conflicts of interest

There are no conflicts of interest to declare.

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