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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis of piperine–amino acid ester conjugates and study of their cytotoxic activities against human cancer cell lines

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Abstract A series of piperine–amino acid ester conjugates (**4a–4r**) were synthesized under mild conditions and screened for their cytotoxic activities against a panel of human cancer cell lines (IMR-32, MCF-7, PC-3, DU-145, Colo-205, and Hep-2). The parent compound piperine lacked significant activity but the analogues were effective to in all tested human cancer cell lines. The introduction of D- and L-amino acid side chain to piperine through peptide linkage significantly increased cytotoxic activity. Among the tested conjugates, **4p** showed significant cytotoxic activity against DU-145 cell lines with IC_{50} of 21 μM. The synthetic protocol is suitable for generating piperine derivatives with various structural motifs for exploring the desired activity.

Keywords *Piper nigrum* · Piperine · Piperoic acid · Amino acid conjugates · Amide linkage · Peptide linkage · Boric acid · Cytotoxic activity

Introduction

Cancer is one of the most serious threats against human health in the world. Recent statistical data showed that it engulf approx eight million people around the world for the year 2007 (Shewach, 2009). Over the past few decades, extensive research has led to the development of a plethora of chemotherapeutic agents; however, none of these agents are capable of completely eliminating cancer (Cragg and Newman, 2005; Cragg *et al*., 2009). The limitations of current anticancer drugs and rapid development of drug resistance (Sreedhar and Csermely, 2004; Pechan, 1991; McCubrey *et al*., 2006) have highlighted the need for the discovery of new anticancer agents, preferably with novel mechanisms of action. To identify new chemical entities for a more effective treatment of cancer, drug designers can follow many strategies, but the crucial decision is always the selection of a suitable starting point from the vast chemical space (Lloyd *et al*., 2006).

In this respect, natural products can be viewed as evolved privileged structures (Koehn and Carter, 2005) and biologically prevalidated leads, in other words, as molecules that have probably evolved evolutionarily to exert highly specialized functions. Recent review pointed out that, about 74% of anticancer compounds being either natural or natural product-derived products, indicating potency of these scaffolds (Newman *et al*., 2003). The unprecedented structures of these molecules make them excellent synthetic targets, and their potent activity against a broad number of therapeutic indications makes these natural products excellent drug lead candidates for new therapeutics (Newman, 2008).

In connection with recent our investigations of *piper* species for value-added products (Sumathykutty and Rao, 1991; Reddy *et al.,* 2004; Srinivas and Rao, 1999; Srinivas *et al.,* 2006; Rao *et al*., 2009; Jyothi *et al*., 2009), we have isolated large quantities of piperine (**1**) (2%) from *Piper nigrum*, which prompted us to synthesize derivatives and screen for the anticancer activity. Traditionally, pepper

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has been used for many ailments particularly as anti inflammatory agent and piperine has been identified as the active principle of pepper (Parmar *et al*., 1997). Piperine (**1**), a hydrophobic amide derived from the fruit of *Piper nigrum* has been shown to exhibit a wide spectrum of biological and pharmacological activities (Mujumdar *et al*., 1990; Birkmayer *et al*., 1985; Kong *et al*., 2004; Pradeep and Kuttan, 2002; Sunila and Kuttan, 2004; Bezerra *et al*., 2006; Selvendran *et al*., 2005). Despite the prominent potential of piperine, very little effort has been devoted into the synthesis of piperine derivatives (Sangwan *et al*., 2008; Nargotra *et al*., 2009; Mishra *et al*., 2005).

Recently, development of hybrid or conjugate molecules between two different types of moieties emerged as a new approach in the discovery of new cytotoxic agents, as they consists of high potency and also different alkylation sites and these properties are essential to play vital role in tumor treatment (Kamal *et al*., 2005; Noh *et al*., 2009). Thus, we wish to utilize amino acids as the diversity surrogates to couple with the piperine. Such piperine–amino acid derivatives may have the advantages of structural diversity, improved solubility, and tunable anticancer activity. In addition, the chemical processes and reactions involved are concise and straightforward, which make it feasible for the scale-up production of piperine derivatives of interest. Based on the consideration of simple processes and ample diversity, we derived a concise strategy for synthesizing piperine–amino acid ester conjugates through the peptide linkage. Herein, we report the synthesis and cytotoxic activity of this new series of derivatives.

Chemistry

Results and discussion

The basic skeleton of piperine consists of methylenedioxyphenyl (MDP) moiety, side chain which consists of conjugated *trans*-double bonds and basic six-membered piperidine moiety attached to side chain via amide linkage. This study aimed to explain SAR studies on basic amide moiety (–CO–N–) in piperine, by replacing with peptide bond (–CO–NH–) through the conjugation of amino acid ester. Thus, the target compounds **4a**–**4r** were prepared through one step condensation of piperic acid (**2**) and amino acid esters (D and L) (**3a**–**3r**) using ecofriendly boric acid in catalytic amount at reflux temperature in toluene (Table 1) (Tang *et al*., 2005). In this reaction, as shown in Scheme 1, the acid moiety of **2** gets activated with boric acid, which in turn provides an electron-deficient acid carbonyl center that reacts with amine (**3a**–**3r**) to afford amide (**4a**–**4r**) in 70–88% yield. Piperic acid (**2**) was obtained through basic hydrolysis of piperine. The acid functionality in all amino acids (D and L) was protected as its methyl ester by following literature method (Medina and EI-Sayed, 2009). However, these approaches of combining different active molecules present in nature, resulting in enhancement of their bioavailability, are certainly encouraging. These molecules contain the unique properties like chirality, hydrophilicity/hydrophobicity, and optical properties (Frank-Furt and Krishnan, 2003; Thiantanwat *et al*., 2000). The distinctive internal composition created by the amino acid building blocks offers stereoselective sites for noncovalent interactions with guest/drug molecule. All target compounds (**4a**–**4r**) were characterized by ^{1}H, ^{13}C NMR, IR, and HREIMS analyses.

Biological activity

Discussion

The in vitro growth inhibition and cytotoxicity assay was performed by NCI according to well-established procedures (Skehan *et al*., 1990). Initially, all the synthesized derivatives (**4a**–**4r**) along with the piperine were screened in a one dose primary anticancer screen for their cytotoxic activity against neuroblastoma (IMR-32), breast (MCF-7), prostate (PC-3, DU-145), colon (Colo-205), liver (Hep-2) cell lines using 5-flurouracil, adriamycin, and mitomycin C as positive controls. As expected, most of the derivatives showed good cytotoxic activity better than piperine itself. Table 2 summarizes their IC_{50} values against panel of human cancer cell lines. From these data, we found that activity was enhanced when the L-amino acids were conjugated to piperine instead of D-amino acids. The amino acids which possess isopropyl, isobutyl, and methyl side chains, such as D-valine, L-leucine, L-isoleucin, and L-alanine, were conjugated to piperine; its cytotoxic activity was dramatically increased to over 10–90%. Especially, the inhibitory activity of **4p**, which possess the heterocyclic side chain such as histidine showed highest growth inhibition (91%) toward the prostate cell line and its IC_{50} value was 21 $\pm$ 0.09. However, it should also note that the parent compound piperine itself is inactive against the prostate cancer cells. It is important to note that compound **4a** displayed moderate activity toward neuroblastma, breast, and prostate cancer cell lines. It is important to note that cell specificity was observed in compound **4p** against prostate cell lines, which showed selective cytotoxicity against DU-145, but inactive toward PC-3, suggesting the imidazole ring structure may contribute to the binding. Similarly, compounds **4g** and **4h** displayed moderate activity on breast cancer and colon cancer cell lines. It is noteworthy to mention that among the tested monobasic aminoacid esters (**3a**–**3h**), compounds **4a**, **4h** showed moderate activity on PC-3 and

Table 1 Structure, reaction time, and yield of conjugates (**4a–4r**)

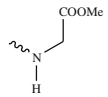
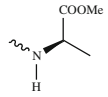
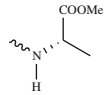
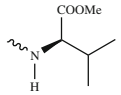
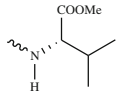
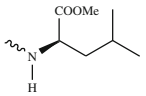
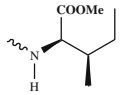
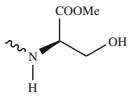
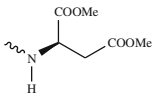
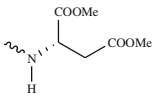
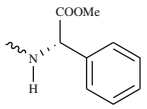
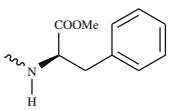
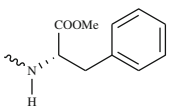
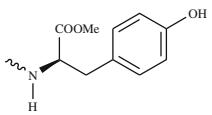
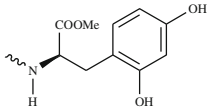
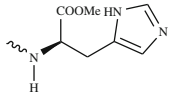
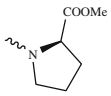
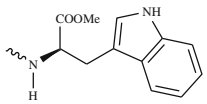
S. no	(**4a-4r**)[a]	R	Time (h)	Yield (%)
1	**a**		6	75
2	**b**		4	80
3	**c**		4	82
4	**d**		6	85
5	**e**		6	86
6	**f**		4	88
7	**g**		4	87
8	**h**		6	80
9	**i**		6	80
10	**j**		5	75
11	**k**		6	75
12	**l**		6	80
13	**m**		6	80
14	**n**		6	75
15	**o**		6	70

Table 1 continued

S. no	(**4a-4r**)[a]	R	Time (h)	Yield (%)
16	**p**		4	85
17	**q**		4	80
18	**r**		4	88

Yield refers to pure products after chromatography

[a] All products were characterized by ^{1}H, ^{13}C NMR, IR and HRE-SIMS spectroscopy

Colo-205 with IC_{50} (33 ± 0.14, 36 ± 0.11) cells. Moreover, the compound **4q** which possesses amide linkage is showed inactive against all cell lines. Overall, these results imply that the mode of perception as well as the structure–activity relationship of the piperine analogues differs considerably between cell lines examined in this study. To the best of our knowledge this is the first report on the anticancer activity of the piperine–amino acid ester conjugates.

Conclusion

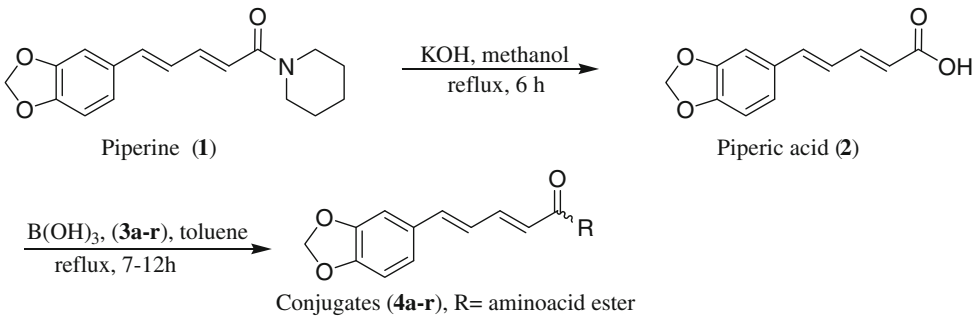
In conclusion, we have observed that the introduction of an amino acid side chain to the piperine moiety causes a significant increase in the cytotoxic activity. Esters of aliphatic L-amino acids were found to be more effective than those of aromatic amino acids. Analogues with hetero aromatic side chain were also good cytotoxic agents. In this study compound **4p** was found to be potent among the tested compounds**.** This initial library represents our early stage effort in diversifying piperine derivatives for exploring useful structure–activity relationships. On the basis of the results observed histidine and glycine could be identified as the best possible side chain among the amino acids screened against the cancer cell lines. By conjugating with amino acids, the reported piperine derivatives manifest improved solubility in various solvents including water.

Experimental

General

Optical rotations were recorded on a JASCO DIP 300 digital polarimeter at 25°C. IR spectra were recorded on

Scheme 1 Synthesis of piperine–amino acid ester conjugates



3a = Methyl ester of glycine
3b = Methyl ester of (L)-alanine
3c = Methyl ester of (D)-alanine
3d = Methyl ester of (L)-valine
3e = Methyl ester of (D)-valine
3f = Methyl ester of (L)-leucine
3g = Methyl ester of (L)-isoleucine
3h = Methyl ester of (L)-serine
3i = Methyl ester of (L)-aspartic acid
3j = Methyl ester of (D)-aspartic acid
3k = Methyl ester of (D)-phenyl glycine
3l = Methyl ester of (L)-phenyl alanine
3m = Methyl ester of (D)-phenyl alanine
3n = Methyl ester of (L)-tyrosine
3o = Methyl ester of (L)-2,4-Dihydroxy phenyl alanine
3p = Methyl ester of (L)-histidine
3q = Methyl ester of (L)-proline
3r = Methyl ester of (L)-tryptopan

Table 2 IC_{50} (μM) for selected conjugates with piperine against human cell lines

Compound	IMR-32 Neuroblastoma	MCF-7 Breast	PC-3 Prostate	DU-145	COLO-205 Colon	HEP-2 Liver
IC-50 (μM)						
4a	50 ± 0.35	44 ± 0.33	33 ± 0.14	49 ± 0.21	–	–
4b	–	–	–	51 ± 0.19	–	–
4d	–	51 ± 0.33	49 ± 0.32	44 ± 0.17	–	–
4e	–	–	–	–	51 ± 0.23	–
4f	–	48 ± 0.42	–	–	55 ± 0.12	43 ± 0.11
4g	–	48 ± 0.35	–	–	–	–
4h	–	–	–	–	36 ± 0.11	–
4i	–	–	–	46 ± 0.13	–	–
4p	–	–	–	21 ± 0.09	–	–
Piperine	89 ± 0.39	99 ± 0.41	–	–	46 ± 0.13	70 ± 0.1
5-FU	nd	2.9 ± 0.3	nd	nd	2.9 ± 0.09	2 ± 0.3
Adriamycin	0.02 ± 0.12	0.8 ± 0.01	nd	nd	nd	nd
Mitomycin C	nd	nd	1.4 ± 0.11	1.8 ± 0.3	nd	Nd

The values are represented as mean ± SE of three experiments for each carried out in triplicate

– >50 μM

nd not determined

Nicolet-740 spectrometer with KBr Pellets. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-300 MHz spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C, respectively, using TMS as internal standard. The chemical shifts are expressed as (δ) values in parts per million (ppm) and the coupling constant (J) is given in hertz (Hz). Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra were measured on LC-MSD-Trap-SL instrument. Melting points were recorded on Electrothermal 9100 and are uncorrected. Most of the reaction solvents were purified by distillation under nitrogen from the indicating drying agent and used fresh, dichloromethane (calcium hydride), acetone (potassium permanganate). Column chromatography was carried out using silica gel 100–200 mesh (Acme Silica gel) and precoated silicagel plates (Merck, 60 F254) were used for preparative TLC. $B(OH)_3$, L and D-amino acids were purchased from Sigma Chemical Co., St Louis, MO, USA. Other chemicals of analytical grade were procured from indigenous manufacturers.

Extraction and isolation of piperine from *Piper nigrum*

The fruits of *Piper nigrum* also known as black pepper were purchased from the local super market, Secunderabad (Andhra Pradesh, India) in the month of December 2007 and identification was made by Department of Botany, Osmania University, Hyderabad. A voucher specimen was deposited at the herbarium of Indian Institute of Chemical Technology, Hyderabad, India. The shade dried fruits of *Piper nigrum* were powdered in a pulvarizer (5 kg) and extracted with hexane in a soxhlet apparatus for 72 h. The resulting extract was filtered to get 10 g of piperine.

Cell lines and cell cultures

The human cancer cell lines were obtained either from National Center for Cell Science, Pune, India or National Cancer Institute, Frederick, USA. The human neuroblastoma (IMR-32), breast (MCF-7), prostate (PC-3, DU-145), colon (colo-205), liver (Hep-2) cell lines were grown and maintained in RPMI-1640medium, pH 7.4, whereas DMEM was used for neuroblastoma (IMR-32) and liver (Hep-2). The media were supplemented with FCS (10%), penicillin (100 U/ml), streptomycin(100 g/ml), glutamine (2 mM) and cells were grown in CO_2 incubator (Heraeus, GmbH, Germany) at 37°C with 90% humidity and 5% CO_2. Cells were treated with A001 and F002 dissolved in DMSO, while the untreated control cultures received only the vehicle (DMSO, <0.2%).

Determination of in vitro cytotoxicity against human cancer cell lines

In vitro cytotoxicity against human cancer cell lines was determined using sulphorhodamine B assay (Skehan *et al*., 1990). Stock solutions of all the compounds were prepared in DMSO and serially diluted with growth medium to obtain desired concentrations.

Experimental procedure for synthesis of **4a**–**4r**

The reaction vessel is charged with piperic acid **2** (0.050 g, 1 mmol), boric acid (0.020 g, 0.30 mmol), and 50 ml of toluene, Dean-Stark trap topped with a reflux condenser. To the stirred colorless reaction mixture is added amino-acid ester (**3a**–**3r**) (1 mmol) in one portion. The reaction mixture is heated at reflux for 12–15 h. The progress of the reaction was monitored by TLC using 40% ethyl acetate–hexane system. The mixture is allowed to cool to ambient temperature and then is poured with stirring into 100 ml of hexane leading to the immediate precipitation of a white solid. Stirring is continued for an additional 15 min and then the precipitate is filtered off with suction through a sintered glass filter funnel. The collected solid, which was purified by column chromatography (40% ethyl acetate–hexane) to give target compounds (**4a**–**4r**). Reaction time and yields of products (**4a**–**4r**) are given in Table 1.

Synthesis of piperic acid (**2**) from piperine (**1**)

Piperine (5 g, 17.5 mmol) was refluxed with methanolic KOH (2 N, 20 ml) for 6 h and methanol was evaporated under reduced pressure, solution cooled in ice bath, the gummy potassium salt of piperic acid was suspended in water and gradually acidified with dilute hydrochloric acid, dark yellow precipitate was collected and stirred for 3–4 h at cold ice bath, filtered through filter paper, and washed with cold water (2 × 10 ml) and recrystallised from methanol to afford yellow crystalline compound with yield of 80%, final compound was submitted to spectral analysis and the spectral values and mp were matched with reported data.

Spectroscopic data of conjugates (**4a**–**4r**)

*Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)acetate (**4a**)*

It is a pale white solid, mp. 152–154°C; IR (KBr) υ_{max}: 663, 808, 993, 1036, 1289, 1489, 1605, 1742, 2853, 2922, 3066, and 3272 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.78 (3H, s), 5.13–5.17 (2H, d, $J = 5.0$ Hz), 5.95–6.02 (1H, d, $J = 14.7$ Hz), 5.98 (2H, s), 6.09–6.18 (1H, br t, $J = 4.9$ Hz), 6.62–6.73 (1H, dd, $J = 15.4$, 10.5 Hz), 6.74–6.83 (2H, m), 6.86–6.91 (1H, dd, $J = 8.1$, 1.7 Hz), 6.95–6.9 (1H, d, $J = 1.5$ Hz), 7.33–7.42 (1H, dd, $J = 14.9$, 10.5 Hz), ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.6, 41.4, 52.3, 101.4, 105.8, 108.5, 121.9, 122.7, 124.4, 130.6, 139.5, 142.0, 148.1, 166.2, 170.4; HRESIMS *m/z* 290.1019 [$M^+ + H$], calcd for $C_{15}H_{15}NO_5$ 290.09502.

*(S)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)propanoate (**4b**)*

It is a yellow solid, mp. 158–160°C; $[\alpha]_D^{25} = +9$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 770, 1039, 1250, 1495, 1649, 1740, 2851, 2919, and 3455 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 1.43–1.48 (3H, d, $J = 7.1$ Hz), 3.77 (3H, s), 4.66–4.77 (1H, m), 5.92–5.97 (1H, d, $J = 13.0$ Hz), 5.98 (2H, s), 6.10–6.17 (1H, br d, $J = 6.98$ Hz), 6.63–6.73 (1H, dd, $J = 15.4$, 10.3 Hz), 6.75–6.82 (2H, m), 6.88–6.92 (1H, dd, $J = 8.1$, 1.5 Hz), 6.99 (1H, d, $J = 1.3$ Hz), 7.31–7.42 (1H, dd, $J = 14.9$, 10.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$):

δ 18.6, 48.1, 52.3, 101.2, 105.7, 108.4, 122.3, 122.7, 124.4, 130.8, 139.1, 141.7, 148.2, 148.3, 165.4, 173.6; HRESIMS *m/z* 304.1180 [M^+ + H], calcd for $C_{16}H_{17}NO_5$ 346.1179.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)propanoate (**4c**)*

It is a yellow solid, mp. 150–154°C; $[\alpha]_D^{25} = -15$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 927, 1030, 1249, 1450, 1538, 1652, 1740, 2852, 2920, and 3457 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 1.42–1.49 (3H, d, $J = 7.1$ Hz), 3.77 (3H, s), 4.66–4.78 (1H, m), 5.92–5.94 (1H, d, $J = 13.0$ Hz), 5.95–6.00 (2H, br s), 6.11–6.18 (1H, br d, $J = 6.98$ Hz), 6.62–6.73 (1H, dd, $J = 15.4$, 10.3 Hz), 6.74 –6.82 (2H, m), 6.87–6.92 (1H, dd, $J = 8.1$, 1.5 Hz), 6.97–6.99 (1H, d, $J = 1.3$ Hz), 7.32–7.42 (1H, dd, $J = 14.9$, 10.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 18.5, 47.1, 51.7, 100.1, 107.9, 109.4, 123.3, 123.6, 125.5, 131.8, 139.1, 141.6, 148.3, 148.4, 165.5, 173.7; HRESIMS *m/z* 304.1181 [M^+ + H], calcd for $C_{16}H_{17}NO_5$ 346.1179.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-methylbutanoate (**4d**)*

It is a white solid, mp. 140–142°C; $[\alpha]_D^{25} = +39$ (*c* 0.50, $CHCl_3$);IR (KBr) υ_{max}: 670, 746, 807, 989, 1039, 1194, 1504, 1645, 1741, 2875, 2961, and 3292 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 0.89–1.00 (6H, m), 2.12–2.26 (1H, m), 3.75 (3H, s), 4.62–4.74 (1H, m), 5.90–6.08 (4H, m), 6.63–6.74 (1H, dd, $J = 15.4$, 10.3 Hz), 6.75–6.85 (2H, m), 6.87–6.93 (1H, dd, $J = 8.1$, 1.3 Hz), 6.97–7.00 (1H, br s), 7.32–7.43 (1H, dd $J = 15.0$, 10.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.9, 18.9, 31.5, 52.1, 57.1, 101.4, 105.7, 108.5, 122.6, 124.5, 130.8, 139.2, 140.9, 148.2, 165.9, 172.7; HRESIMS *m/z* 332.1490 [M^+ + H], calcd for $C_{18}H_{21}NO_5$ 332.1492.

*(S)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-methylbutanoate (**4e**)*

It is a white solid, mp. 139–141°C; $[\alpha]_D^{25} = +45$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 758, 930, 1037, 1251, 1497, 1653, 1738, 2358, 2963, and 3304 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 0.91–0.95 (3H, d, $J = 6.7$ Hz), 0.95–1.00 (3H, d, $J = 6.9$ Hz), 2.13–2.27 (1H, m), 3.76 (3H, s), 4.65–4.73 (1H, m), 5.96–5.99 (3H, m), 5.95–6.01 (1H, d, $J = 14.9$ Hz), 6.62–6.72 (1H, dd, $J = 15.4$, 10.5 Hz), 6.74–6.81 (2H, m), 6.81–6.91 (1H, dd, $J = 8.1$, 1.3 Hz), 6.97 (1H, br d, $J = 1.3$ Hz), 7.31–7.41 (1H, dd $J = 14.9$, 10.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 17.8, 18.9, 31.5, 52.1, 57.0, 101.3, 105.7, 108.5, 122.4, 122.6, 124.4, 130.7, 139.3, 141.8, 148.2, 148.3, 165.8, 172.6; HRESIMS *m/z* 332.1492 [M^+ + H], calcd for $C_{18}H_{21}NO_5$ 332.1492.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-4-methylpentanoate (**4f**)*

It is a pale yellow solid, mp. 111–115°C; $[\alpha]_D^{25} = +20$ (*c* 0.10, $CHCl_3$); IR (KBr) υ_{max}: 866, 1037, 1200, 1444, 1536, 1653, 1740, 2857, 2921, and 3414 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 0.95 (6H, t, $J = 5.2$ Hz), 1.53–1.65 (2H, m), 1.67–1.76 (1H, m), 3.75 (3H, s), 4.72–4.82 (1H, m), 5.92–6.00 (4H, m), 6.62–6.72 (1H, dd, $J = 15.8$, 10.5 Hz), 6.74–6.81 (2H, m), 6.87–6.92 (1H, dd, $J = 8.3$, 1.5 Hz), 6.97–6.99 (1H, d, $J = 1.5$ Hz), 7.33–7.42 (1H, dd, $J = 15.1$, 10.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 22.0, 22.7, 24.9, 29.6, 41.9, 50.7, 52.3, 101.3, 105.7, 108.4, 122.3, 122.8, 124.6, 130.7, 139.4, 141.9, 148.2, 165.7, 173.7; HRESIMS *m/z* 346.1637 [M^+ + H], calcd for $C_{19}H_{23}NO_5$ 346.1649.

*(2R,3R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-methylpentanoate (**4g**)*

It is a pale yellow solid, mp. 112–115°C; $[\alpha]_D^{25} = +68$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 805, 929, 1037, 1255, 1444, 1534, 1644, 1741, 2964, and 3303 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 0.91–0.98 (6H, t, $J = 7.1$ Hz), 1.40–1.54 (1H, m), 1.88–1.98 (1H, m), 3.76 (3H, s), 4.70–4.76 (1H, dd, $J = 8.6$, 5.0 Hz), 5.99 (2H, s), 5.95–6.01 (1H, d, $J = 14.7$ Hz), 6.02–6.07 (1H, br d, $J = 9.0$ Hz), 6.63–6.73 (1H, dd, $J = 15.4$, 10.3 Hz), 6.76–6.82 (2H, m), 6.88–6.93 (1H, dd, $J = 8.1$, 1.7 Hz), 6.99 (1H, d, $J = 1.5$ Hz), 7.32–7.42 (1H, dd, $J = 14.9$, 10.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 11.5, 15.3, 25.2, 38.1, 53.0, 56.3, 101.2, 105.6, 108.4, 122.5, 122.6, 124.4, 130.6, 139.2, 141.7, 148.1, 165.9, 172.7; HRESIMS *m/z* 346.1643 [M^+ + H], calcd for $C_{19}H_{23}NO_5$ 346.1649.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-hydroxypropanoate (**4h**)*

It is a yellow solid, mp. 148–150°C; $[\alpha]_D^{25} = +60$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 663, 1263, 1644, 1731, 2361, 2854, 2925, and 3450 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 2.70 (1H, br s), 3.80 (3H, s), 4.05 (2H, m), 4.80 (1H, m), 5.95 (2H, s), 6.55 (1H, br d, $J = 7.5$ Hz), 6.75 (1H, d, $J = 15.0$ Hz), 6.80 (2H, m), 6.85 (1H, dd, $J = 8.5$, 1.3 Hz), 6.95 (1H, d, $J = 1.3$ Hz), 7.35–7.45 (1H, dd, $J = 14.9$, 10.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 52.7, 54.9, 63.9, 101.6, 105.5, 108.2, 121.9, 122.5, 124.5, 128.6, 130.8, 139.8, 142.3, 166.3, 170.8; HRESIMS *m/z* 320.1113 [M^+ + H], calcd for $C_{16}H_{17}NO_6$ 320.1129.

*(R)-Dimethyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)succinate (**4i**)*

It is a yellow solid, mp.154–156°C; $[\alpha]_D^{25} = +26$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 999, 1248, 1345, 1495, 1649, 1738, 2851, 2920, and 3450 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 2.88–2.98 (1H, dd, $J = 17.3$, 4.5 Hz), 3.06–3.15 (1H, dd, $J = 17.3$, 4.1 Hz), 3.70 (3H, s), 3.78 (3H, s), 4.94–5.01 (1H, m), 5.95 (NH, br s), 5.98–6.00 (2H, s), 6.54–6.59 (1H, d, $J = 7.9$ Hz), 6.3–6.74 (1H, dd, $J = 15.4$, 10.5 Hz), 6.76–6.83 (2H, m), 6.88–6.93 (1H, dd, $J = 8.1$, 1.5 Hz), 6.98–7.00 (1H, d, $J = 1.5$ Hz), 7.34–7.43 (1H, dd, $J = 14.9$, 10.3 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.1, 48.6, 51.9, 52.8, 101.3, 105.7, 108.5, 122.1, 122.7, 124.5, 130.7, 139.6, 142.0, 148.2, 148.3, 165.6, 171.2, 171.6; HRESIMS *m/z* 362.1234 [$M^+ + H$], calcd for $C_{18}H_{19}NO_7$ 362.1234.

*(S)-Dimethyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)succinate (**4j**)*

It is a white solid, mp.150–152°C; $[\alpha]_D^{25} = -20$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 850, 929, 1036, 1153, 1246, 1367, 1493, 1597, 1648, 1734, 2336, 2920, 3044, 3288, and 3729 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 2.88–2.97 (1H, dd, $J = 17.3$, 4.5 Hz), 3.06–3.15 (1H, dd, $J = 17.3$, 4.1 Hz), 3.70 (3H, s), 3.78 (3H, s), 4.92–5.01 (1H, m), 5.94–6.01 (3H, m), 6.54–6.59 (1H, br d, $J = 7.9$ Hz), 6.63–6.74 (1H, dd, $J = 15.4$, 10.5 Hz), 6.77–6.85 (2H, m), 6.88–6.93 (1H, d, $J = 8.1$, 1.7 Hz), 6.99 (1H, d, $J = 1.7$ Hz), 7.33–7.43 (1H, dd, $J = 14.9$, 10.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 36.1, 48.6, 51.9, 52.8, 101.3, 105.7, 108.5, 122.1, 122.7, 124.5, 130.7, 139.6, 142.0, 148.2, 148.3, 165.6, 171.2, 171.6; HRESIMS *m/z* 362.1236 [$M^+ + H$], calcd for $C_{18}H_{19}NO_7$ 362.1234.

*(S)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-2-phenylacetate (**4k**)*

It is a pale yellow solid, mp. 165–167°C; $[\alpha]_D^{25} = -41$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 809, 929, 1040, 1255, 1369, 1496, 1529, 1644, 1736, 2782, 2890, 3026, and 3311 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.75 (3H, s), 5.67–5.72 (1H, d, $J = 7.3$ Hz), 5.94–6.03 (1H, d, $J = 14.6$ Hz), 5.98 (2H, s), 6.49–6.66 (1H, dd, $J = 15.4$, 10.2 Hz), 6.72–6.76 (2H, m), 6.80 (1H, br s), 6.87–6.92 (1H, dd, $J = 8.0$, 2.2 Hz), 6.96–6.99 (1H, d, $J = 1.4$ Hz), 7.30–7.44 (6H, m). ^{13}C NMR (75 MHz, $CDCl_3$): δ 52.8, 56.4, 101.4, 105.6, 108.6, 122.0, 122.7, 124.5, 127.2, 128.5, 129.0, 130.6, 136.7, 139.7, 142.1, 148.0, 148.3, 165.3, 171.4; HRESIMS *m/z* 366.1340 [$M^+ + H$], calcd for $C_{21}H_{19}NO_5$ 366.1336.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-phenylpropanoate (**4l**)*

It is a yellow solid, mp. 118–120°C; $[\alpha]_D^{25} = +148$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 807, 927, 1036, 1196, 1348, 1441, 1533, 1646, 1735, 2338, 2946, 3027, 3305, and 3733 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.16–3.23 (2H, t, $J = 5.3$ Hz), 3.75 (3H, s), 4.98–5.05 (1H, m), 5.88–5.95 (1H, d, $J = 14.9$ Hz), 5.96–6.01 (3H, m) 6.62–6.74 (1H, dd, $J = 15.4$, 10.7 Hz), 6.76–6.86 (2H, m), 6.87–6.93 (1H, dd, $J = 8.3$, 1.8 Hz), 6.97–7.03 (1H, dd, $J = 9.6$, 1.7 Hz), 7.27–7.31 (5H, m), 7.32–7.42 (1H, dd, $J = 14.9$, 10.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.9, 52.3, 53.2, 101.3, 105.7, 108.5, 122.2, 122.6, 124.4, 126.9, 128.5, 129.2, 130.6, 135.8, 139.4, 141.9, 148.2, 165.5, 172.1; HRESIMS *m/z* 380.1482 [$M^+ + H$], calcd for $C_{22}H_{21}NO_5$ 380.1492.

*(S)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-phenylpropanoate (**4m**)*

It is a yellow solid, mp. 123–125°C; $[\alpha]_D^{25} = -28$ (*c* 0.25, $CHCl_3$); IR (KBr) υ_{max}: 811, 997, 1036, 1149, 1251, 1369, 1494, 1532, 1652, 1741, 2853, 2921, 3029, and 3299 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.16–3.22 (2H, t, $J = 5.4$ Hz), 3.75 (3H, s), 4.96–5.05 (1H, m), 5.88–5.94 (1H, d, $J = 14.7$ Hz), 5.91–5.96 (NH, br d, $J = 7.5$ Hz), 5.99 (2H, br s), 6.62–6.72 (1H, dd, $J = 15.4$, 10.5 Hz), 6.77–6.80 (1H, d, $J = 8.3$ Hz), 6.76–6.82 (1H, d, $J = 15.4$ Hz), 6.88–6.92 (1H, dd, $J = 8.1$, 1.7 Hz), 6.98 (1H, d, $J = 1.5$ Hz), 7.08–7.13 (2H, dd, $J = 7.9$, 1.8 Hz), 7.24–7.34 (3H, m), 7.32–7.42 (1H, dd, $J = 14.7$, 10.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 37.9, 52.3, 53.2, 101.3, 105.7, 108.5, 122.1, 122.7, 124.3, 127.0, 128.6 (2), 128.7, 129.2 (2), 130.9, 135.8, 139.5, 141.8, 165.4, 172.0; HRESIMS *m/z* 380.1485 [$M^+ + H$], calcd for $C_{22}H_{21}NO_5$ 380.1492.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-(4-hydroxyphenyl)propanoate (**4n**)*

It is a yellow solid, mp. 157–160°C; $[\alpha]_D^{25} = +54$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 804, 924, 1035, 1148, 1348, 1443, 1599, 1646, 1720, 2895, 2949, 3026, and 3374 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.03–3.19 (2H, m), 3.75 (3H, s), 4.93–5.00 (1H, m), 5.88–5.95 (1H, d, $J = 14.9$ Hz), 5.98–5.99 (2H, br s), 5.99–6.02 (1H, br d, $J = 7.5$ Hz), 6.61–6.71 (1H, dd, $J = 15.4$, 10.5 Hz), 6.72 –6.74 (1H, br s), 6.85–6.78 (2H, m), 6.79–6.82 (1H, m), 6.88–6.92 (1H, dd, $J = 8.1$, 1.5 Hz), 6.93–6.99 (3H, m), 7.32–7.41 (1H, dd, $J = 14.9$, 10.5 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.7, 52.2, 53.2, 96.2, 101.4, 105.6, 108.6, 115.4, 122.3, 122.7, 124.5, 130.0, 139.5, 142.0, 148.3,

151.6, 154.8, 165.5, 172.1; HRESIMS *m/z* 396.1451 [M^+ + H], calcd for $C_{22}H_{21}NO_6$ 396.1442.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-(2,4-dihydroxyphenyl)propanoate (**4o**)*

It is a yellow solid, mp. 140–142°C; IR (KBr) υ_{max}: 809, 928, 1037, 1116, 1250, 1444, 1604, 1732, 2852, 2922, and 3361 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.08–3.18 (2H, m), 3.73 (3H, s), 4.89–5.03 (1H, m), 5.89–6.03 (4H, m), 6.06–6.20 (1H, m), 6.62–7.06 (7H, m), 7.28–7.41 (1H, dd, $J = 14.5$, 10.7 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 32.5, 52.1, 56.3, 100.8, 101.4, 105.2, 105.8, 108.9, 118.2, 120.9, 121.9, 126.1, 131.4, 131.9, 136.5, 140.4, 147.4, 147.9, 154.8, 156.8, 165.2, 171.9; HRESIMS *m/z* 412.1375 [M^+ + H], calcd for $C_{22}H_{21}NO_7$ 412.1391.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-(1H-imidazol-5-yl)propanoate (**4p**)*

It is a yellow solid, mp. 135–137°C; $[\alpha]_D^{25} = +32$ (*c* 0.75, $CHCl_3$); IR (KBr) υ_{max}: 811, 925, 1036, 1146, 1251, 1489, 1596, 1655, 1700, 1741, 1845, 2361, 2897, and 3624 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.05–3.24 (2H, m), 3.75 (3H, s), 5.00 (1H, m), 5.90–6.08 (4H, m), 6.69–6.93 (4H, m), 6.96–7.09 (3H, m), 7.32–7.40 (1H, dd, $J = 14.5$, 10.3 Hz), 8.19 (NH, br s). ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.6, 52.0, 52.5, 101.2, 105.7, 108.4, 113.9, 116.1, 122.6, 124.6, 136.0, 139.1, 141.6, 144.3, 148.1, 150.0, 161.5, 165.9, 171.8; HRESIMS *m/z* 370.1398 [M^+ + H], calcd for $C_{19}H_{19}N_3O_5$ 370.1397.

*(R)-Methyl-1-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienoyl)pyrrolidine-2-carboxylate (**4q**)*

It is a pale yellow solid, mp. 115–117°C; $[\alpha]_D^{25} = +48$ (*c* 1.0, $CHCl_3$); IR (KBr) υ_{max}: 929, 1038, 1250, 1444, 1643, 1740, 2852, and 2921 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$): δ ppm 1.86–2.31 (4H, m), 3.55–3.72 (2H, m), 3.74 (3H, s), 4.50–4.64 (1H, m), 5.97 (2H, s), 6.20–6.35 (1H, d, $J = 14.6$ Hz), 6.71–6.84 (3H, m), 6.87–6.95 (1H, dd, $J = 8.0$, 1.4 Hz), 6.98–7.01 (1H, br s), 7.38–7.53 (1H, dd, $J = 14.6$, 10.2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.8, 29.2, 46.8, 52.1, 59.0, 101.3, 105.6, 108.4, 120.3, 122.7, 125.0, 130.8, 139.3, 143.0, 148.2, 165.2, 172.8; HRESIMS *m/z* 330.1335 [M^+ + H], calcd for $C_{18}H_{19}NO_5$ 330.1336.

*(R)-Methyl-2-((2E,4E)-5-(benzo[d][1,3]dioxol-6-yl)penta-2,4-dienamido)-3-(1H-indol-3-yl)propanoate (**4r**)*

It is a yellow solid, mp. 118–120°C; $[\alpha]_D^{25} = +111$ (*c* 0.50, $CHCl_3$); IR (KBr) υ_{max}: 805, 927, 1036, 1149, 1250, 1490, 1606, 1652, 1738, 2361, 2950, and 3289 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ ppm 3.34–3.41 (2H, m), 3.68–3.70 (3H, s), 5.02–5.10 (1H, m), 5.80–5.88 (1H, d, $J = 15.1$ Hz), 5.96–5.98 (2H, br s), 6.09–6.17 (1H, d, $J = 7.5$ Hz), 6.56–6.67 (1H, dd, $J = 15.1$, 10.5 Hz), 6.71–6.84 (2H, m), 6.85–6.91 (1H, d, $J = 8.3$ Hz), 6.95–6.99 (1H, d, $J = 1.5$ Hz), 7.04–7.23 (3H, m), 7.25–7.40 (2H, m), 7.51–7.57 (1H, d, $J = 7.5$ Hz), 8.26–8.31 (1H, br s). ^{13}C NMR (75 MHz, $CDCl_3$): δ 27.7, 52.4, 53.1, 101.3, 105.7, 108.5, 110.5, 111.2, 118.6, 119.6, 122.1, 122.3, 122.7, 122.9, 124.4, 127.6, 130.7, 136.1, 139.4, 141.7, 148.2, 165.6, 172.3; HRESIMS *m/z* 419.1607 [M^+ + H], calcd for $C_{24}H_{22}N_2O_5$ 419.1601.

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