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Synthesis and Erythropoietin Receptor Binding Affinities of *N,N*-Disubstituted Amino Acids

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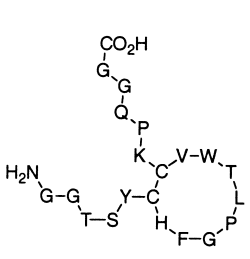
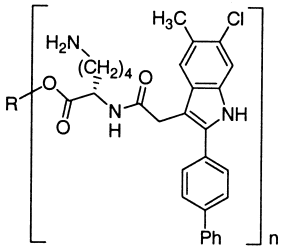
Abstract—*N,N*-Dicinnamyl, *N*-benzyl-*N*-cinnamyl, and *N,N*-dibenzyl amino acids were prepared and evaluated in an EPO binding assay. Several derivatives of aspartic acid, glutamic acid, and lysine exhibited moderate (10–50 μM) affinity for EBP; 'dimerization' of the most potent analogues by coupling with linear diamines led to EPO competitors having 1–2 μM binding affinities.

Introduction

Erythropoietin (EPO) is a 34 kD glycoprotein hormone that is the principal growth factor responsible for the regulation of red blood cell production in humans.[1] Over the past decade, recombinant human EPO has become a mainstay in the treatment of anemias resulting from chronic renal failure, chemotherapy, and HIV-infection. Because of the inconvenience and expense of therapy involving an injectable protein like EPO, there is considerable interest in an orally available, small molecule EPO mimetic. However, designing a nonprotein molecule with the ability to mimic the complex interaction between a protein hormone and its receptor has proved to be challenging. Literature references to small molecule antagonists of cytokine receptors (i.e., small molecule inhibitors of interleukin-2 binding to its receptor[2]) predominate. On the other hand, the past three years have witnessed reports of nonprotein cytokine receptor agonists such as a nonpeptide mimic of granulocyte-colony stimulating factor[3] and an EPO receptor-derived peptide that activates receptor signalling.[4] Previously, our group had disclosed a series of cyclic peptides, exemplified by EMP1 (**1**), possessing potent EPO-mimetic activity.[5,6] A recent publication describing a nonpeptide molecule having EPO-mimetic activity (**2b**, derived from EPO receptor antagonist **2a**)[7] prompted us to disclose our work toward EPO-mimetic small molecules.



1 EMP1



2a n = 1 R = CH_3
2b n = 8 R = Starburst dendrimer

Synthesis and Biological Results

Based on our experience with EPO-mimetic peptides and the desire to explore easily accessible chemistry, we synthesized a series of *N,N*-dicinnamyl amino acids and their corresponding esters for screening in an immobilized EPO receptor (EBP) binding assay (Scheme 1).[8] Starting from side-chain-protected amino acid esters **3** (β- or γ-*t*-butyl ester for aspartic and glutamic acid, *t*-butyl ether for serine, ε-Boc for lysine), alkylation with the appropriately substituted cinnamyl bromide[9] resulted in easily separated mixtures of di- and monoalkylated amino esters (**4** and **5**; X = *trans*-CH=CHCH$_2$). The purified mono- and dicinnamyl compounds were tested directly in the EBP binding assay or were optionally hydrolyzed to the respective amino acids using reagents appropriate for the nature of the ester (50% TFA/CH_2Cl_2 for α-*t*-butyl esters; NaOH for α-methyl esters). *t*-Butyl and *N*-Boc side-chain protecting groups were removed using 50% TFA/CH_2Cl_2, and the lysine ε-amino

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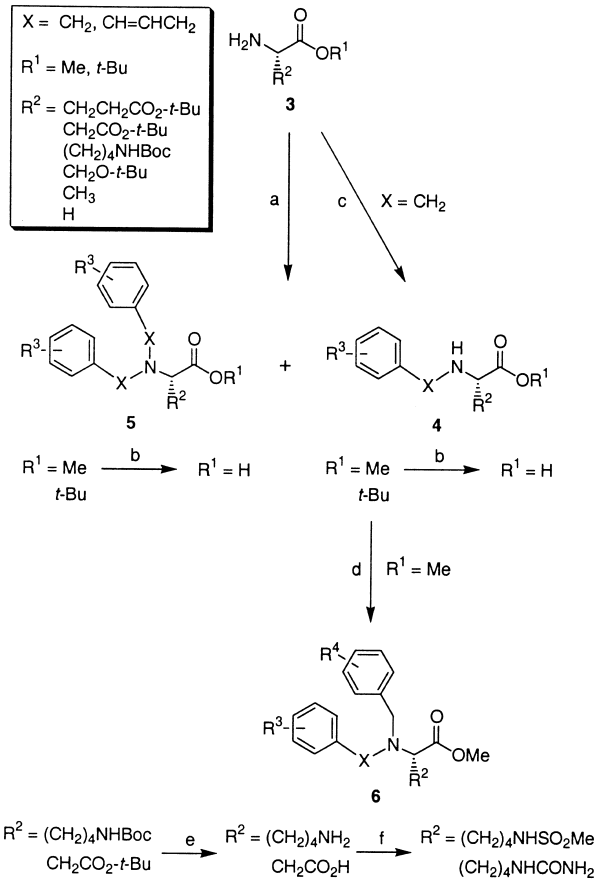


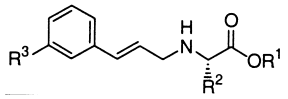
Scheme 1. (a) $R^3C_6H_4$-X-Br or $R^3C_6H_4$-X-Cl/NaI, DIEA, DMF; (b) 50% TFA/CH_2Cl_2 (R^1 = *t*-Bu) *or* aqueous NaOH (R^1 = Me); (c) $R^3C_6H_4$-CHO, $NaBH(OAc)_3$, $(MeO)_3CH$; (d) $R^4C_6H_4$-CH_2Br *or* $R^4C_6H_4$-CH_2Cl/NaI, DIEA, DMF; (e) 50% TFA/CH_2Cl_2; (f) MsCl, Et_3N *or* TMS-NCO, Et_3N.

group was further converted to the corresponding urea or sulfonamide.

Many of the substituted amino acid derivatives were only weakly competitive with ^{125}I-EPO at EBP (Tables 1 and 2). In particular, the monocinnamyl amino acids and esters (**4**) were uniformly inactive, as were dicinnamyl derivatives (**5**) that contained protected side-chain functional groups. However, significant binding affinities ($IC_{50} < 50\,\mu M$) were observed for dicinnamyl compounds having a free carboxylate, with the most potent analogues coming from the aspartic/glutamic acid family (**5b**, **5d**, **5e**, and **5g**). Alanine derivative **5o** and lysine derivatives having polar substituents on the side-chain nitrogen (**5i** and **5j**) were also potent. Interestingly, the free ε-amino compound (**5l**) was inactive.

Additional aspartic acid and lysine analogues were synthesized using a modification of the route described above (Scheme 1). To prepare *N*-benzyl-*N*-cinnamyl derivatives, *N*-cinnamyl amino acid esters **4** (X = *trans*-$CH{=}CHCH_2$) were reacted with DIEA and an appropriately substituted benzyl halide in DMF. Treatment of the resulting side-chain-protected products with TFA in CH_2Cl_2 provided **6** (X = *trans*-$CH{=}CHCH_2$). Symmetrically substituted *N,N*-dibenzyl analogues **5** (X = CH_2) were prepared by alkylation of amino acid esters **3** with

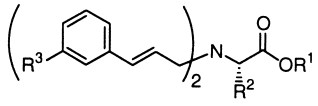
Table 1.



Compound	R^1	R^2	R^3	EBP binding[a] % inh, 50 μM	EBP binding[a] IC_{50}, μM
4a	Me	$CH_2CH_2CO_2$-*t*-Bu	PhO	6	nd[b]
4b	H	$CH_2CH_2CO_2$-*t*-Bu	PhO	0	nd
4c	Me	$CH_2CH_2CO_2H$	PhO	6	nd
4d	H	$CH_2CH_2CO_2H$	PhO	0	nd
4e	Me	$(CH_2)_4NHBoc$	PhO	20	nd
4f	Me	CH_2O-*t*-Bu	PhO	8	nd
4g	Me	CH_2OH	PhO	28	nd
4h	H	CH_2OH	PhO	23	nd

[a]Inhibition of ^{125}I-EPO binding to EBP.[8]
[b]nd = Not determined.

Table 2.



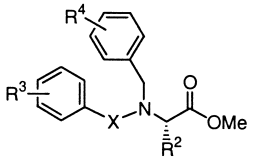
Compound	R^1	R^2	R^3	EBP binding[a] % inh, 50 μM	EBP binding[a] IC_{50}, μM
5a	Me	$CH_2CH_2CO_2$-*t*-Bu	PhO	0	nd[b]
5b	H	$CH_2CH_2CO_2H$	PhO	78	42
5c	Me	$CH_2CH_2CO_2H$	3,4-Cl_2PhO	32	nd
5d	H	$CH_2CH_2CO_2H$	4-*t*-Bu-PhO	78	30
5e	H	$CH_2CH_2CO_2H$	3-CF_3-PhO	70	35
5f	*t*-Bu	CH_2CO_2-*t*-Bu	PhO	20	nd
5g	H	CH_2CO_2H	PhO	81	36
5h	Me	$(CH_2)_4NHBoc$	PhO	0	nd
5i	Me	$(CH_2)_4NHCONH_2$	PhO	73	35
5j	Me	$(CH_2)_4NHSO_2Me$	PhO	77	35
5k	Me	$(CH_2)_4NHBoc$	4-*t*-Bu-PhO	0	nd
5l	Me	$(CH_2)_4NH_2$	4-*t*-Bu-PhO	18	nd
5m	Me	CH_2O-*t*-Bu	PhO	0	nd
5n	Me	CH_2OH	PhO	57	145
5o	H	CH_3	PhO	72	40
5p	H	H	PhO	45	85

[a]Inhibition of ^{125}I-EPO binding to EBP.[8]
[b]nd = Not determined.

substituted benzyl halides. This reaction also provided the corresponding mono-*N*-benzyl derivatives **4** (X = 6;CH_2) which, after separation, were further alkylated with other benzyl halides to give unsymmetrically substituted *N,N*-dibenzyl analogues **6** (X=CH_2). The intermediate mono-*N*-benzyl amino acid esters **4** (X=CH_2) were alternatively prepared by reductive alkylation using a substituted benzaldehyde, $(MeO)_3CH$, and $NaBH(OAc)_3$.[10]

Several *N,N*-dibenzyl- and *N*-benzyl-*N*-cinnamyl derivatives (**6a**, **6b**, and **6d–f**) were similar in potency to the best dicinnamyl compounds in the EPO competition assay (Table 3). However, inhibition of EPO binding at the screening dose of 50 μM was only modest, indicating relatively flat dose–response curves. Once again, side-chain-protected compounds (**6c** and **6h–j**) were inactive,

Table 3.



Compound	X	R^2	R^3	R^4	EBP binding[a]	
					% inh, 50 μM	IC_{50} μM
6a	$CH{=}CHCH_2$	$(CH_2)_4NH_2$	3-(4-*t*-Bu-PhO)	3-BnO	46	10
6b	$CH{=}CHCH_2$	$(CH_2)_4NH_2$	3-(PhO)	3-PhO	60	30
6c	$CH{=}CHCH_2$	$(CH_2)_4NHBoc$	3-(4-*t*-Bu-PhO)	3-BnO	0	nd[b]
6d	$CH{=}CHCH_2$	CH_2CO_2H	3-(PhO)	3-PhO	52	40
6e	CH_2	$(CH_2)_4NH_2$	3-(BnO)	3-BnO	59	10
6f	CH_2	$(CH_2)_4NH_2$	4-(BnO)	4-BnO	54	10
6g	CH_2	$(CH_2)_4NH_2$	4-(BnO)	3-NO_2	37	nd
6h	CH_2	$(CH_2)_4NHBoc$	3-(BnO)	3-BnO	7	nd
6i	CH_2	$(CH_2)_4NHBoc$	4-(BnO)	4-BnO	5	nd
6j	CH_2	$(CH_2)_4NHBoc$	4-(BnO)	3-NO_2	24	nd

[a]Inhibition of ^{125}I-EPO binding to EBP.[8]
[b]nd = Not determined.

but the free ε-amino lysine analogues were fairly potent competitors, in contrast to the poor affinity exhibited by **5l**.

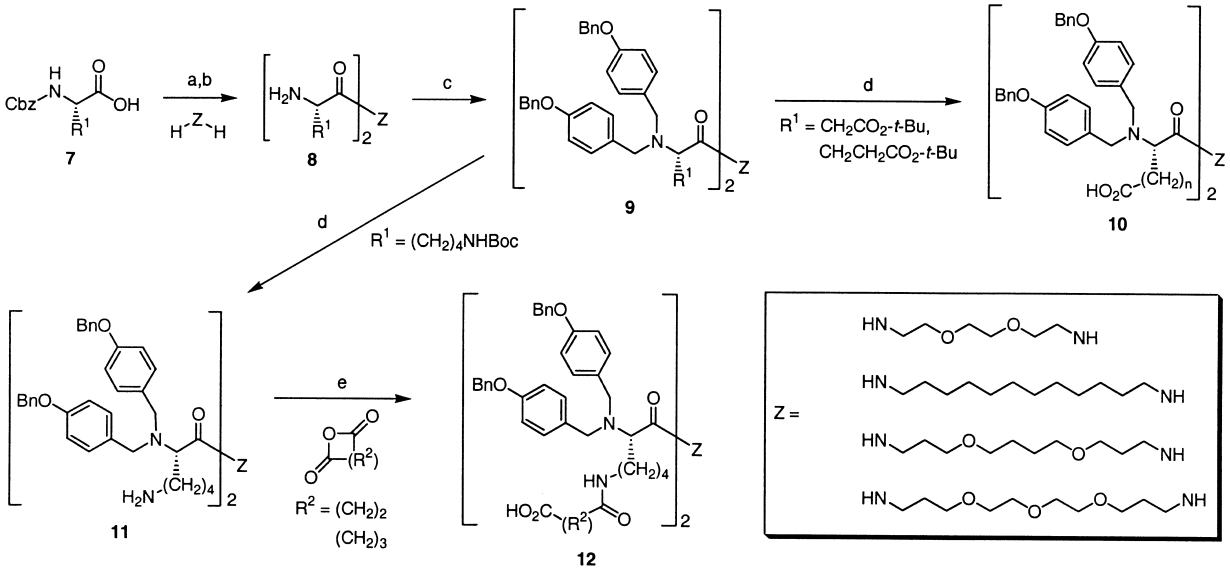
In an effort to improve binding affinity, we next prepared 'dimeric' analogues in which two moderately potent EPO competitors were connected by a hydrocarbon or polyether linking group. For our purposes, a series of commercially available diamines proved to be a convenient set of reagents, allowing us to explore the effects of distance and linker hydrophobicity on EBP affinity. Because of the relative potency of benzyloxybenzyl derivatives **6a**, **6e**, and **6f** (Table 2), we chose to make 'dimeric' analogues of *N,N*-di-(4-benxyloxybenzyl) amino acids. Toward that end, 2 equiv of N_α-Cbz-protected aspartic acid (β-*t*-butyl ester), glutamic acid (γ-*t*-butyl ester), or lysine (N_ε-Boc) (**7**) were coupled with the appropriate diamine using water-soluble carbodiimide (EDCI) and HOBt/DIEA in CH_2Cl_2 (Scheme 2). The Cbz group was removed by standard or catalytic transfer hydrogenation; the resulting diamino intermediate **8** was exhaustively *N*-alkylated with 4-benzyloxybenzyl chloride and sodium iodide in DMF. After purification, the side-chain-protected compound **9** was treated with TFA in CH_2Cl_2 to provide the respective tetrabenzyl derivative **10** (Asp or Glu) or **11** (Lys). For the lysine series, the side-chain amino group was further derivatized by reaction with succinic or glutaric anhydride to provide **12**.

Discussion

As was mentioned previously, EBP binding of 'monomeric' amino acid analogues **4**–**6** was affected by both the degree of substitution at the α-nitrogen atom and by the nature of the substituents on the side chain (Tables 1–3). While all of the mono-*N*-cinnamyl derivatives were essentially inactive at 50 μM, many side-chain-protected dicinnamyl derivatives were equally weak EPO competitors. Aspartic and glutamic acid analogues were also inactive unless both the α- and ω-carboxylates were present as free acids (**5b**, **5d**, **5e**, and **5g**). In the lysine series, side-chain deprotection was also necessary for EBP binding affinity (**6a**, **6b**, **6e**, and **6f**), although there was no apparent need for a free α-carboxy group. Interestingly, conversion of the ε-amino group to a urea (**5i**) or methanesulfonamide (**5j**) was accompanied by moderately potent EBP binding affinity, suggesting that polar functionality, rather than positive charge, was beneficial. In order to determine whether the compounds possessed EPO-mimetic activity, the highest affinity analogues were evaluated in an EPO-responsive FDC-P1 cell proliferation assay but, unlike EMP1 (**1**), none were found to be active.[5]

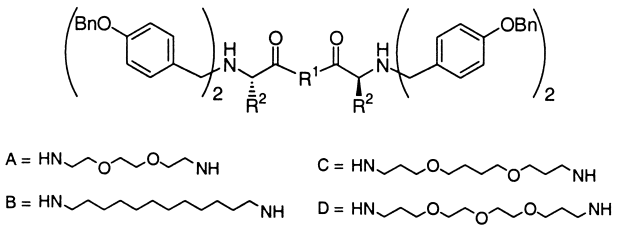
It is known that EPO exerts its effect at the molecular level by bringing two molecules of EPO receptor together on the cell surface. 'Dimeric' targets **10**–**12** were designed to exploit this property by linking together two EPO competitors with the appropriate tether, in theory converting a small molecule antagonist into an EPO-mimetic receptor agonist. In practice, tethered compounds **10**–**12** were highly effective EPO competitors, especially in the lysine series, with many having IC_{50} values below 10 μM (Table 4). Linker length (11–20 Å between N atoms in the extended conformation) and composition (hydrophobic vs hydrophilic) affected binding affinity, with the most potent derivatives **12a** and **12e** ($IC_{50} = 1.5$ μM) arising from the shortest hydrophilic linker, $HNCH_2CH_2(OCH_2CH_2)_2NH$. The hydrophobic linker, $HN(CH_2)_{12}NH$, was associated with the lowest affinity 'dimers' (**12b** and **12f**), possibly due to poor access to the EPO binding site resulting from hydrophobic collapse of the linker.

Unfortunately, despite EPO receptor binding affinity on par with that of EPO-mimetic peptide EMP1 (**1**), the best 'dimeric' analogues (**11**, **12a**, **12c–e**, **12g**, and **12h**) did not promote proliferation in the FDC-P1 cell assay.[5] Apparently, the tethered construct exemplified



Scheme 2. (a) EDCI, HOBt, DIEA, CH_2Cl_2; (b) Pd–C, H_2 *or* Pd–C, NH_4CO_2, MeOH; (c) 4-$BnOC_6H_4CH_2Cl$, NaI, DMF; (d) 50% TFA/CH_2Cl_2; (e) DMAP, CH_2Cl_2.

Table 4.



Compound	R^1	R^2	EBP binding[a] % inh, 50 μM	EBP binding[a] IC_{50}, μM
10a	C	CH_2CO_2H	75	18
10b	D	CH_2CO_2H	66	28
10c	A	$CH_2CH_2CO_2H$	79	75
11	A	$(CH_2)_4NH_2$	51	2
12a	A	$(CH_2)_4NH\text{-}CO(CH_2)_3CO_2H$	88	1.5
12b	B	$(CH_2)_4NH\text{-}CO(CH_2)_3CO_2H$	55	85
12c	C	$(CH_2)_4NH\text{-}CO(CH_2)_3CO_2H$	82	4
12d	D	$(CH_2)_4NH\text{-}CO(CH_2)_3CO_2H$	78	10
12e	A	$(CH_2)_4NH\text{-}COCH_2CH_2CO_2H$	93	1.5
12f	B	$(CH_2)_4NH\text{-}COCH_2CH_2CO_2H$	56	100
12g	C	$(CH_2)_4NH\text{-}COCH_2CH_2CO_2H$	65	6
12h	D	$(CH_2)_4NH\text{-}COCH_2CH_2CO_2H$	77	7
1		EMP1[6]	—	5

[a]Inhibition of ^{125}I-EPO binding to EBP.[8]

by **10–12** was not flexible enough or could not span the distance required to bring together two EPO receptor molecules. The additional binding affinity gained by the 'dimer' construct was likely due to added nonspecific hydrophobic interactions outside the EPO binding pocket of EBP or the EPO receptor.

In conclusion, moderate EBP binding affinity was observed for *N,N*-disubstituted analogues of lysine, aspartic acid, and glutamic acid, with a preference for acidic or polar residues on the amino acid side chains. Coupling of two *N,N*-dibenzyl derivatives to a hydrophilic diamine produced 'dimers' like **12a** and **12e** having 10-fold greater binding affinity. Unfortunately, neither the 'dimers' nor the corresponding parent compounds exhibited EPO-mimetic activity in a cell proliferation assay.

References and Notes

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