Pergamon

Tetrahedron Letters 43 (2002) 5339–5341

TETRAHEDRON LETTERS

A microbial metabolite inhibitor of CD28–CD80 interactions

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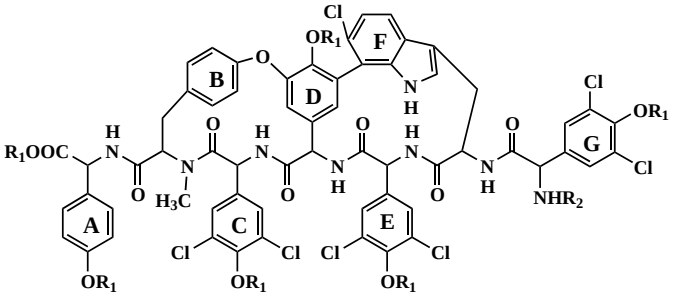
Received 19 April 2002; accepted 17 May 2002

Abstract—The organic extract of the fermentation broth of a *Streptomycete* microorganism was found to contain SCH 212394 (**1**), a condensed aromatic peptide. The structure was established by extensive NMR spectral data and derivatization. SCH 212394 (**1**), inhibited CD28–CD80 costimulation with an IC_{50} of 1.25 μM. © 2002 Published by Elsevier Science Ltd.

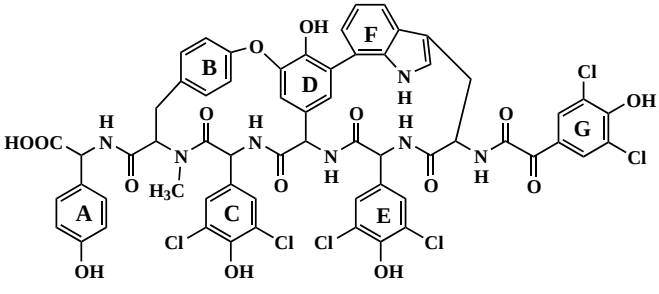
CD28 is a T-cell costimulatory receptor, which plays a pivotal role in antigen-induced T-cell response.[1] CD28 costimulation enhances T-cell responses to antigens and also can prevent activated T cells from entering states of anergy and apoptosis.[2,3] In contrast to signaling of the T-cell receptor, which is sensitive to calcineurin inhibitors such as cyclosporine A or FK506, cellular signaling of CD28 was shown to be resistant to calcineurin inhibitors.[4–6] This notion is supported by the high efficacy of CTLA-4Ig, a potent competitor of CD28 in binding to CD80 or CD86 in treating animals suffering from autologous transplant rejection and experimental autoimmune diseases. Thus CD28 costimulation pathway may provide alternative targets of pharmacological intervention for transplant rejection and experimental autoimmune diseases.[7,8] To search for novel inhibitors of CD28–CD80 costimulation, we developed a high throughput screen based on ligand–receptor interactions.

As part of our continuing investigation of natural products as leads for treating autoimmune diseases, we screened ethyl acetate extracts of several microbial fermentation broths. Fermentation broth of a microorganism belonging to *Streptomycete* sp. was identified as displaying distinct activity in the CD28/80 assay.[9] Bioassay guided fractionation of this extract led to the isolation of **1**.

A 5 L fermentation broth was adjusted to pH 3.0 with dil. HCl and extracted twice with 10 L of ethyl acetate. The organic layers were combined, dried over anhydrous Na_2SO_4, and the solvent removed to yield 860 mg of solids. These solids were loaded onto a MCI CHP-20P (2.5×25 cm) column and eluted with acetonitrile and 0.05% trifluoroacetic acid gradient. The fractions were monitored for activity and for purity using the CD28 assay and HPLC (Water's Deltapak column, 0.46×25 cm, mobile phase: acetonitrile:0.05% trifluoroacetic acid



1. $R_1 = H$, $R_2 = H$
2. $R_1 = CH_3$, $R_2 = H$
3.. $R_1 = CH_3$, $R_2 = COCH_3$



2. Chloropeptin I

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PII: S0040-4039(02)01029-8

(35:65). The active fractions were collected and dried to yield 128 mg of enriched complex. The compound was further purified by reverse-phase preparative HPLC on a Water's Deltapak C-18 silica column (1.9×30 cm), eluting with a mixture of acetonitrile and 0.05% trifluoroacetic acid (35:65). Acetonitrile was removed from the peak eluate, and the aqueous solution was freeze-dried to yield 34.6 mg pure SCH 212394 (**1**).

SCH 212394 (**1**) showed a molecular ion cluster from m/z 1361–1370 in the FAB mass spectrum, which suggested the presence of several halogen atoms in the molecule. Analysis of this mass ion cluster suggested SCH 212394 contained seven chlorine atoms. The molecular formula of **1** was established to be $C_{61}H_{47}N_8O_{14}Cl_7$ by HRMS (obsd 1363.1171 calcd. for 1363.1037). The UV spectrum (MeOH) displayed maxima at 245 and 290 nm and the IR spectrum in KBr (pellet) showed peaks at 3300, 1650, 1500, 1490 cm^{-1}, suggesting the presence of amide and ester functionalities. ^1H and ^{13}C NMR chemical shifts of **1** are shown in Table 1. The ^{13}C NMR showed 61 carbon signals which is in agreement with the established molecular formula. The ^1H NMR indicated the presence of one methyl, two methylenes, seven α-amino acid carbon type methines, and many aromatic carbons. APT ^{13}C NMR identified them as seven >C=O, 19 aromatic =CH–, 25 aromatic =C<, seven >CH–N, two $>CH_2$ and one $-CH_3$. The methyl signal appeared to be *N*-methyl group. These results suggested that this compound must be an aromatic condensed peptide similar to those of the chloropeptin family.[10,11] Our previous work with chloropeptin I[12] helped us establish the structure of this compound. The molecular formula of chloropeptin I is $C_{61}H_{45}N_7O_{15}Cl_6$. SCH 212394 (**1**) contains an additional two protons, a nitrogen and a chlorine but lacks one oxygen atom in comparison to chloropeptin I. Table 1 shows ^1H and ^{13}C NMR chemical shifts of **1** and chloropeptin I (**2**), respectively. Comparison of proton and carbon chemical shifts of **1** and **2** revealed them to be very similar compounds except in the terminal group containing phenyl ring **G**. In chloropeptin it is 2-(3,5-dichloro-4-hydroxyphenyl)-2-oxoacetic acid or a deaminated 3,5-dichloro-4-hydroxyphenylglycine forming a keto-amide. Compound **1** appears to contain a 3,5-dichloro-4-hydroxyphenylglycine residue. This was confirmed by derivatization of **1**. Compound **1**, upon methylation with diazomethane afforded a hexamethyl derivative **3** (FABMS m/z 1445), and compound **3** upon acetylation with pyridine/acetic

Table 1. ^1H and ^{13}C NMR chemical shifts of **1** and **2**

^{13}C **1**	^1H **2**	^{13}C **1**	^1H **2**	^{13}C **2**	Carbon no.	^1H **1**	^{13}C **1**	^1H **2**	^{13}C **2**
A. 4-Hydroxyphenylglycine					C_3		150.4		150.6
C=O		171.6		171.5	-C_4		143.8		141.8
α	4.97 (d, 6 Hz)	56.3	5.04 (d, 6.5 Hz)	55.9	-C_5		123.5		126.2
-NH-	8.54 (d, 8 Hz)		8.41 (d, 6.5 Hz)		-C_6	5.56	125.6	5.99 (d, 2.0 Hz)	125.9
βC_1		126.1		127.8	*E. 3,5-Dichloro-4-hydroxyphenylglycine*				
C_2, C_6	7.13 (d, 8.0 Hz)	128.8	7.08 (d, 8.5 Hz)	128.4	C=O		168.7		169.0
C_3, C_5	6.77 (d, 8.0 Hz)	115.2	6.74 (d, 8.5 Hz)	115.4	α	5.42	52.9	5.41 (d, 8.5 Hz)	53.9
-C_4		157.3		157.3	-NH-	8.23		8.19 (d)	
B. N-Methyltyrosine					βC_1		132.0		132.2
C=O		169.0		168.6	C_2, C_6		126.2	7.28	126.7
α	5.07 (dd, 12.4 Hz)	61.2	5.06 (d)	61.5	C_3, C_5	7.12	121.7		121.9
β	3.05	35.5	3.02 (m)	35.1	-C_4		148.0		148.2
-NCH_3	3.06	31.2	2.99 (s)	31.2	*F. Tryptophan*				
γ-C_1		133.5		134.1	C=O		168.5		169.3
C_2	7.72	131.3	7.19 (dd, 8, 2 Hz)	130.3	Cα	5.04	54.7	5.08 (m)	54.9
C_3	6.63	122.5	6.79 (dd, 8, 2 Hz)	123.0	Cβ	2.90, 3.10	28.9	3.12 (m)	26.7
-C_4		157.3		156.2	NH	8.74		8.90 (d, 6.0 Hz)	
-C_5	7.20	121.0	7.14 (dd, 8, 2 Hz)	121.5	1′	10.50		10.57 (bs)	
-C_6	7.23	129.8	7.82 (dd, 8, 2 Hz)	131.6	2′	7.53	126.6	7.64 (d, 2.0 Hz)	126.0
C. 3,5-Dichloro-4-hydroxyphenylglycine					3′		107.2		107.0
C=O		169.0		169.3	3′a		127.0		129.1
-NH	8.76		8.79 (d, 6.0 Hz)		4′	7.29 (d, 8 Hz)	118.2	7.22 (d, 8.0 Hz)	116.7
α	5.04	51.6	5.16 (d, 6.0 Hz)	51.7	5′	6.95 (d, 8 Hz)	119.8	6.90 (t, 8.0 Hz)	118.7
βC_1		131.4		131.3	6′		125.5	7.08 (d, 8.0 Hz)	120.8
C_2, C_6	7.26	126.6	7.36 (d, 8.5 Hz)	127.2	7′		124.7		125.6
C_3, C_5		121.9		122.1	7′a		137.7		135.6
-C_4		148.4		148.8	*G. 3,5-Dichloro-4-hydroxyphenylglycine*				
D. 3,4-Dihydroxyphenylglycine					C=O		166		164.5
C=O		168.1		168.1	Cα	4.80	54.2		185.4
α	5.56	54.8	5.61 (d, 8.5 Hz)	55.0	βC_1		128.8		127.2
-NH-	8.21		8.25 (d, 8.5 Hz)		C_2, C_6	7.33	128.1	7.82 (s)	130.4
βC_1		126.2		126.4	C_3, C_5		121.9		122.8
C_2	5.93	114.4	5.70 (d, 2.0 Hz)	112.2	-C_4		149.7		157.8

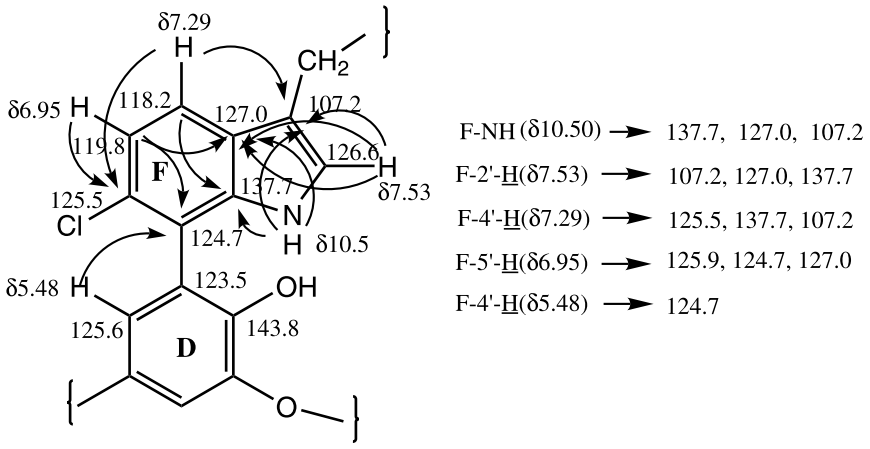

Figure 1. Selected HMBC correlation's to establish the chlorine atom on tryptophan (ring **F**).

anhydride, overnight at room temperature gave an acetyl derivative **4** (FABMS *m/z* 1487). These results confirmed the presence of an amino group between the terminal **G** ring and the amide bond in **1** instead of a keto-amide as in **2**. This accounted for two protons and a nitrogen instead of an oxygen as in **2**. The position of an additional chlorine was established to be at the C-6′ of tryptophan (amino acid F) based on ^{13}C NMR chemical shifts (120.8 in **1**, 125.5 in **2**). In compound **1**, the ^{1}H NMR chemical shift due to C-6′ at δ 7.08 of chloropeptin I was absent and that due to C-5′ appeared as doublet (δ 6.95) instead of triplet (δ 6.90) as in chloropeptin I. The structure was further confirmed by COSY, HETCOR and HMBC. The selective HMBC correlations showing the position of the additional chlorine is shown in Fig. 1. We have not seen any other related compounds from this family during bio-assay guided fractionation of this extract.

The purified SCH 212394 (**1**) showed an IC_{50} of 1.2 μM in the CD28[9] assay in the presence of fetal bovine serum (FBS) and 0.07 μM in its absence. It also showed an IC_{50} of 0.13 μM in the CD4-*gp*120 binding in the absence of FBS and an IC_{50} of 8.9 μM in the complement assay. Chloropeptin I (**2**) was the most potent inhibitor of CD28–CD80 interaction with an IC_{50} of 0.42 μM in the CD28 assay in the presence of FBS and 0.02 μM in its absence and also showed an IC_{50} of 7.9 μM in the complement assay.

Acknowledgements

The authors graciously acknowledge Mr. J. Troyanovich for his support in supplying us with active fermentation broths.

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