Isolation of RP 66453, a New Secondary Peptide Metabolite from *Streptomyces* sp. Useful as a Lead for Neurotensin Antagonists

GERARD HELYNCK*, CATHERINE DUBERTRET, DENISE FRECHET† and JEAN LEBOUL

Rhône-Poulenc Rorer S. A.,
Centre de Recherches de Vitry-Alfortville,
Natural Products Department,
†Structural Analysis Department,
13 Quai Jules Guesde B.P. 14,
94403 Vitry sur Seine Cedex, France

(Received for publication October 6, 1997)

The tridecapeptide neurotensine has been the subject of increased interest since its discovery and characterization in the brain[1]. Aspects of its pharmacology include physiological effects both in the periphery and the central nervous system[2]. In order to elucidate further its pathophysiological role, neurotensin agonists and antagonists are needed.

In the course of our screening program to find substances that displace the neurotensin from its receptor, a receptor binding assay using guinea-pig brain membranes was performed according to GOEDERT *et al.*[3]. During this screening, RP 66453 was isolated as a novel secondary metabolite produced by an *Actinomycetes* strain.

For the production of this compound the *Streptomyces* strain A 9738 was inoculated into a 2 liter Erlenmeyer flask containing 250 ml of seed medium consisting of peptone 0.5%, yeast extract 0.5%, corn steep 0.5%, glucose 1.5%, $CaCO_3$ 0.3%, NaCl 0.5% and agar 0.1%. The seed culture was incubated for 3 days at 28°C on a rotary shaker (140 rpm). The whole culture was transferred into a 100 liter fermentor containing 60 liters of the same medium and incubated at 28°C under agitation (300 rpm) and aeration (5 m^3/hour) for 2 days. The whole culture was finally transferred into an 800 liter fermentor containing 450 liters of a medium consisting of distillers 0.5%, beans 4%, glucose 0.5%, soybean oil 1%, NaCl 0.5% and $CoCl_2$ 0.002% (15 m^3/hour) for 94 hours.

The broth (487 liters, pH 7.8) was complemented with Clarsel (5%), homogeneized under agitation and filtered off to separate supernatant and mycelium. The mycelium was discarded and the supernatant (300 liters) was extracted twice with 100 liters ethyl acetate. The organic phase was discarded and the aqueous phase (250 liters) was ultrafiltered (cut off 20 Kda). The resulting ultrafiltrate (210 liters) was applied on a Duolite S 861 stainless steel column (20 × 60 cm). The column was washed with water (80 liters) and eluted stepwise with a MeOH-H_2O gradient. The active fractions as monitored by binding assay were eluted with 60% MeOH, combined, concentrated under reduced pressure and lyophilized. The resulting lyophilizate (12 g) was extracted by cooled MeOH (600 ml), the insoluble residue was discarded and the solution was concentrated under reduced pressure to yield a crude powder (4.4 g). This powder was further purified by HPLC on an Amicon C18 (20 μm, 60A) column (5 × 60 cm) using an aqueous gradient of increasing methanolic concentration. The active fractions were combined and concentrated under reduced pressure to yield an aqueous solution that was applied on a Macherey-Nagel polyamide CC6 column (5 × 30 cm). The column was eluted with water and the active fractions were combined and applied on a Bio-Rad DEAE

Table 1. Physico-chemical properties of RP 66453.

Appearance	Beige powder
Anal Calcd for $C_{33}H_{36}N_4O_8$	C 64.27, H 5.88, N 9.09
Found	C 64.30, H 6.20, N 9.10
$[\alpha]_D^{20}$	−181.1° (*c* 1, MeOH)
SI-MS (M+H)	617
UV λ_{max}^{MeOH} nm	292
IR (KBr) cm^{-1}	3395, 3300, 2970, 2935, 2875, 1670, 1585, 1500 1235
Rf value[a]	0.7
Solubility	
Soluble	MeOH, DMSO
Insoluble	Hexane, $CHCl_3$, EtOAc

[a] Merck Art. No. 5715, ethyl acetate–acetic acid–water (4:1.2:1).

Fig. 1. Structure of RP 66453.

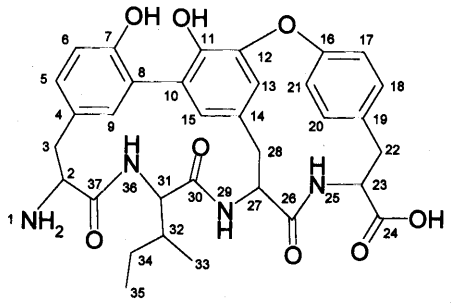
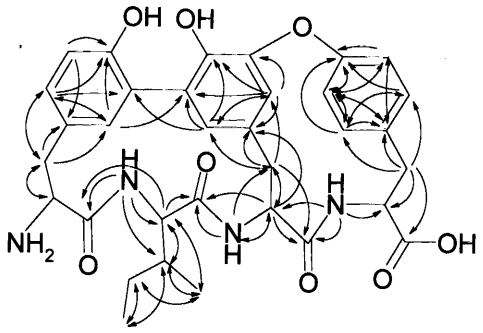


Fig. 2. ^{1}H-^{13}C long range coupling observed in HMBC spectrum of RP 66453.



Biogel A column (5 × 13 cm). This column was again eluted with water and the active fractions containing the pure metabolite were combined and applied on a C18 column (2.5 × 8 cm) that was washed with water and eluted with MeOH. The methanolic solution was taken to dryness under reduced pressure to yield 60 mg of the pure metabolite as a beige powder. The physico-chemical properties of the metabolite RP 66453 are summarized in Table 1. Its structure (Fig. 1) was assigned by NMR studies. The spectral properties of this metabolite suggested it was a peptide. The ^{1}H and ^{13}C NMR spectral data are shown in Table 2. The molecular formula $C_{33}H_{36}N_4O_8$ was derived from ^{1}H, ^{13}C NMR, MS spectra and elemental analysis. ^{1}H-^{1}H COSY and HOHAHA experiments allowed to identify four spin systems corresponding to three modified tyrosine residues and one isoleucine residue. HSQC and HMBC experiments (Fig. 2) indicated that the isoleucine residue was located at the second position in the sequence of this tetrapeptide. C-8 as well as C-10 and C-12 bore no protons. Long range proton-carbon correlations between H-9 and C-10 and between H-15 and C-8 led to the identification of a bond between C-8 and C-10. The ^{13}C

Table 2. ^{1}H and ^{13}C NMR data of RP 66453 in CD_3OH at 600.13 MHz.

Position	δ ^{1}H (ppm)	δ ^{13}C (ppm)
1	broad	—
2	4.10 dd (5.6, 1.0)	54.2
3	2.94 dd (14.5, 5.6)	37.6
	3.35 dd (14.5, 1.0)	
4	—	125.8
5	7.00 dd (8.1, 1.8)	132.3
6	6.77 d (8.1)	117.6
7	—	155.2
8	—	128.6
9	6.89 d (1.8)	137.2
10	—	131.2
11	—	144.7
12	—	154.5
13	5.65 d (1.2)	121.0
14	—	137.5
15	6.67 d (1.2)	126.7
16	—	164.7
17	6.70 dd (8.3, 2.2)	125.2
18	7.09 dd (8.3, 2.2)	135.6
19	—	135.1
20	7.24 dd (8.3, 2.2)	131.3
21	7.33 dd (8.3, 2.2)	127.5
22	2.85 dd (13.2, 7.2)	37.8
	3.49 dd (13.2, 1.0)	
23	4.16 ddd (8.8, 7.2, 1.0)	55.0
24	—	177.3
25	4.46 d (8.8)	—
26	—	173.5
27	3.69 ddd (9.6, 5.6, 3.9)	60.2
28	2.40 dd (13.9, 3.9)	41.8
	3.05 dd (13.9, 5.6)	
29	8.59 d (9.6)	—
30	—	173.6
31	4.06 dd (6.5, 7.5)	62.0
32	1.66 m	37.5
33	0.84 d (6.8)	16.1
34	1.15 m	27.0
	1.50 m	
35	0.80 t (7.3)	11.4
36	8.45 d (6.5)	—
37	—	171.0

The CD_3OH signal was used as reference (^{1}H, 3.2 ppm and ^{13}C, 49.5 ppm). The coupling constants (Hz) are in parentheses.

chemical shifts of C-12 (154.5 ppm) and C-11 (144.7 ppm) as well as the observation of strong NOEs between the aromatic proton H-13 and the four aromatic protons H-17, H-18, H-20, H-21 allowed to identify the biaryl ether bridge between C-12 and C-16. Linking C-11 to C-16 was excluded after molecular modeling studies (steric hindrance). All these data suggested the structure

of RP 66453 being as depicted Fig. 1. It was shown to be an original peptidic secondary metabolite that could be regarded as a constrained *C*-terminal moiety of the 8～13 neurotensin (Arg-Arg-Pro-Tyr-Ile-Leu). It has been shown to bind very specifically to the neurotensin receptor from guinea-pig (IC_{50}, 30 μg/ml) as compared with other receptors such as Neuropeptide Y, Angiotensin II, Galanin *etc.*,... (inactive at 380 μg/ml). Hemisynthesis work on this molecule has led to valuable neurotensin antagonists[4]. While writing this note we became aware that RP 66453 is thought to be identical with Cittilin B that was isolated along with saframycin from *Myxococcus xanthus*. (H. REICHENBACH, personal communication).

References

1) KALIVAS, P.: Neurotransmitter regulation of dopamine neurons in the ventral tegmental area. Brain Res. Rev. 18: 75～113, 1993
2) KITABGI, P.; F. CHECLER, J. MAZELLA & J. P. VINCENT: Pharmacology and biochemistry of neurotensin receptors. Rev. Clin. Basic Pharmacol. 5: 397～486, 1985
3) GOEDERT, M.; K. PITTAWAY, B. J. WILLIAMS & P. C. EMSON: Specific binding of tritiated neurotensin to rat brain membranes: characterization and regional distribution. Brain Research 304: 71～81, 1984
4) CLERC, F. F.; M. C. DUBROEUCQ, G. HELYNCK, J. LEBOUL & J. P. MARTIN (Rhône-Poulenc Rorer S. A.): Neurotensin antagonist peptides useful for treating e.g. psychoses, depression, Alzheimer's and Parkinson's diseases, schizoprenia, ulcers, acute pancreatitis and asthma. FR 2720066, November 24, 1995