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Short communication

A sequential high-yielding large-scale solution-method for synthesis of philanthotoxin analogues

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Abstract

A general, improved procedure for rapid synthesis of philanthotoxin analogues, a pharmacologically important class of polyamine conjugates, is described. The solution-phase procedure is illustrated by gram-scale synthesis of philanthotoxins PhTX-343 and PhTX-12. Selectively protected polyamines are coupled to N^{α}-Fmoc-protected amino acid pentafluorophenyl esters. After removal of the N^{α}-Fmoc group, the amine is coupled with carboxylic acid pentafluorophenyl esters. Deprotection followed by a rapid and efficient purification by vacuum liquid chromatography on octadecylsilyl silica (RP-18 phase) gave the philanthotoxin analogues in 74–78% overall yield.

Keywords: Polyamines; Philanthotoxins; Solution-phase synthesis; Purification

1. Introduction

Venoms of spiders and wasps contain polyamine toxins used to paralyse prey [1,2]. Their analogues comprise a pharmacologically important class of non-competitive inhibitors of ionotropic receptors. Recently, potency at a nanomolar level and selectivity towards various classes of receptors have been demonstrated [1] with certain analogues of philanthotoxin-433, a venom constituent of the Egyptian digger wasp. This emphasises the interest in further studies of structure–activity relationships for which a rapid, efficient and scalable synthetic method is necessary.

The modular structure of philanthotoxins, which comprises an amino acid moiety, an *N*-acyl group and a polyamine, linked via amide bonds, enables easy construction of combinatorial libraries of philanthotoxin analogues. Classical methods of polyamine toxin synthesis, involving coupling of *p*-nitrophenyl esters of *N*-acylamino acids with appropriately protected polyamines, have been developed by Nakanishi et al. [3]. However, this route is not sequential as the *N*-acyl group is introduced at an early stage. A similar approach involving monoacylation of unprotected spermine (**1**, Fig. 1) with *N*-butyryl-L-tyrosine via a dicyclohexylcarbodiimide coupling has been described [4].

Recently, a number of methods for solid-phase synthesis of polyamine toxins have been developed [5–7]. Solid-phase methods are quite convenient for preparation of small amounts of compounds for high-throughput screening. When a particular compound is required in larger amounts for more detailed studies, solid-phase methods become quite expensive due to the cost of the resin and the need of using a large excess of reagents, often repeatedly, to ensure a full conversion. For example, for preparation of ca. 100 mg of a toxin on solid phase, several grams of reagents are typically used. Moreover, if the polyamine moiety is symmetrical as in the case of spermine (**1**) or other α,ω-diamines, a solution-phase strategy seems more appropriate. The majority of philanthotoxin analogues contain spermine (**1**) as the polyamine moiety [3,8], and the interest in analogues having an α,ω-diamine part (e.g. 1,12-diaminododecane) has emerged recently due to their pronounced selectivity for the nicotinic acetylcholine receptors [7].

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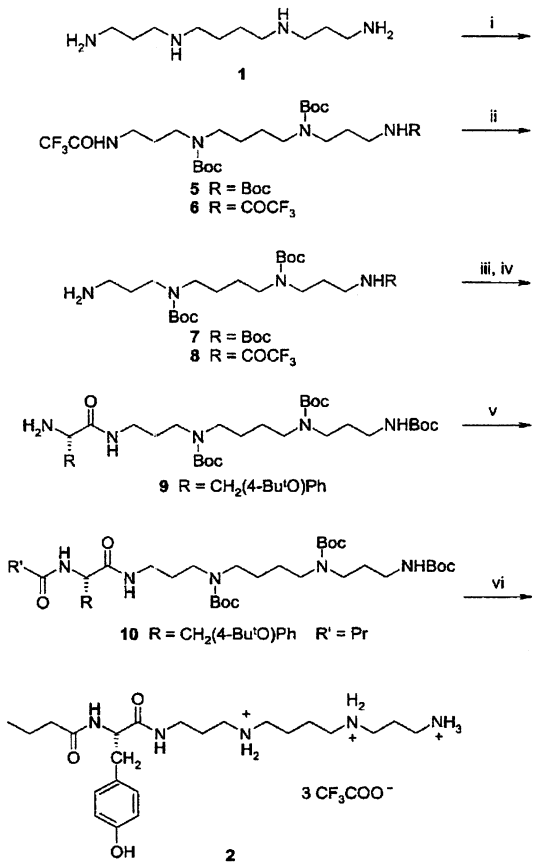
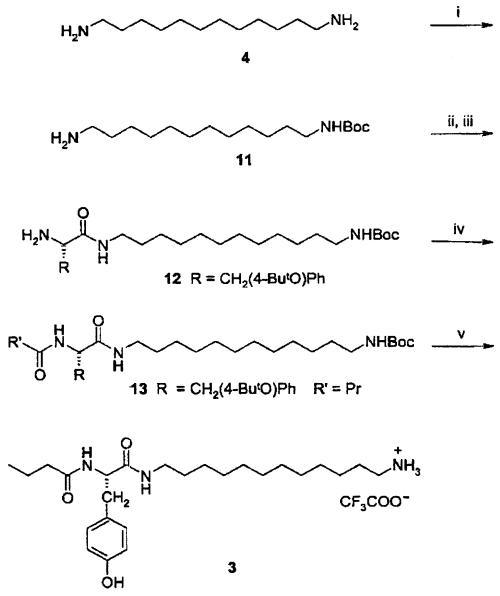
The present work is concerned with the development of improved methods of synthesis as well as purification of polyamine toxins. Such methods should allow convenient parallel synthesis of small combinatorial libraries from readily available and stable building blocks. A sequential strategy similar to that used in solid-phase peptide synthesis was chosen. Purification of the highly polar philanthotoxins is troublesome, and preparative reverse-phase HPLC, used thus far [6,7], is not easily applied for gram-scale purifications.

The pharmacologically important toxins, PhTX-343 (**2**, Fig. 1) (typically employed as a pharmacological reference compound) and PhTX-12 (**3**, Fig. 2) each contain a symmetrical, commercially available polyamines, spermine (**1**) or 1,12-diaminododecane (**4**), respectively. The amino acid unit (Tyr) was selected to be incorporated via the corresponding N^{α}-Fmoc-protected pentafluorophenyl (Pfp) ester, as a vast number of this type of amino acid derivatives are commercially available or can readily be prepared [9,10]. The *N*-acylation



(i) 1 eqv. $F_3CCOOEt$, then 4 eqv. Boc_2O; (ii) NaOH, H_2O– MeOH; (iii) Fmoc-NHCH(R)COOPfp, NEt_3; (iv) DBU, octanethiol–THF; (v) R'COOPfp; (vi) TFA-CH_2Cl_2

Fig. 1. Synthesis of PhTX-343 (**2**).



(i) Boc_2O; (ii) Fmoc-NHCH(R)COOPfp, NEt_3; (iii) DBU, octanethiol-THF; (iv) R'COOPfp; (v) TFA-CH_2Cl_2

Fig. 2. Synthesis of PhTX-12 (**3**).

was likewise envisaged to be effected using an active Pfp-ester of a carboxylic acid.

2. Chemistry

A prerequisite for the chosen strategy is an easy access to large amounts of tri-Boc-protected spermine (**7**). A one-pot literature procedure [11] involving a selective monoprotection of **1** in methanol at low temperature (−78 °C) with a trifluoroacetyl (Tfa) group, followed by Boc-protection of the remaining amino functionalities and subsequent ammonolysis, posed two obstacles. Firstly, a distinct precipitation of **1** was observed upon cooling of the methanolic solution below −50 °C. Secondly, removal of the *N*-trifluoroacetyl groups in **6**, formed as a substantial by-product, was incomplete using methanolic ammonia under the conditions described [11]. This resulted in an inseparable mixture of **7** and **8** (the latter could be detected in the crude reaction product by the presence of ^{13}C-NMR signals at δ 116 and 157, corresponding to trifluoroacetamide, as opposed to trifluoroacetate anion, which appears at δ 118 and 163 (in $CDCl_3$)].

These problems were solved by performing the initial Tfa-protection at −50 °C (±2 °C, manual control) instead of at −78 °C, and by using aqueous sodium hydroxide to remove the Tfa-protecting groups, to give pure **7** in 43% yield. This procedure also avoids the

formation of trifluoroacetamide as a by-product, which turned out to be surprisingly difficult to remove. Coupling of **7** with Fmoc-protected L-tyrosine pentafluorophenyl ester (with the side chain protected as a *tert*-butyl ether) in the presence of one equivalent of triethylamine and subsequent Fmoc-removal using octanethiol–DBU in THF [12] proceeded in high yield. However, the solubility properties of **9** did not allow a facile removal of impurities by a trituration procedure, often feasible for protected peptides [12,13]. Consequently, a purification step was necessary, readily achieved by vacuum liquid chromatography (VLC) on silica gel. Acylation of **9** with pentafluorophenyl butyrate (obtained from the corresponding acid chloride by treatment with pentafluorophenol in the presence of pyridine [14]) and removal of the Boc-protecting groups gave the final product **2**.

VLC using C_{18}-coated silica gel proved to be a very convenient method for purification of gram quantities of **2** in a short time. The final product was, as expected, enantiomerically pure as shown by capillary electrophoresis [6] using heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin as a chiral additive (Fig. 3). Thus, the chirality of the amino acid moiety has been preserved during all synthetic steps. The overall yield of **2** from **7** was 78%.

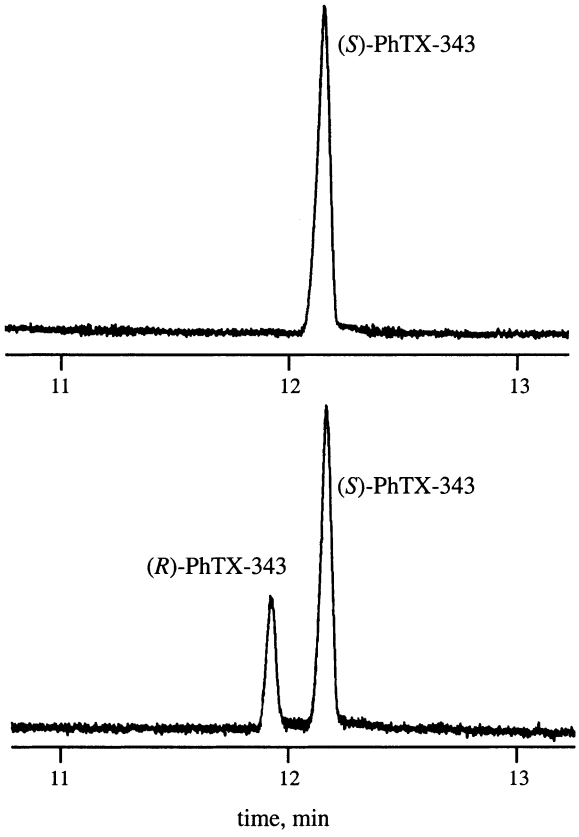


Fig. 3. Capillary electrophoresis of PhTX-343 (**2**) prepared as shown in Fig. 1 (64.5 cm × 50 μm I.D. fused silica capillary (56 cm to detector), 30 kV polarity, 30 mM heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin, 50 mM 6-aminocaproic acid, pH 4.0, 15 °C, detection at 214 nm). Top, (*S*)-PhTX-343 obtained from L-Tyr. Bottom, Mixture of (*S*)- and (*R*)-PhTX-343. (i) 1 equiv. $F_3CCOOEt$, then 4 equiv. Boc_2O; (ii) NaOH, H_2O–MeOH; (iii) Fmoc-NHCH(R)COOPfp, NEt_3; (iv) DBU, octanethiol–THF; (v) R′COOPfp; (vi) TFA–CH_2Cl_2.

To demonstrate the efficiency of this protocol further, a large-scale synthesis of PhTX-12 (**3**) starting from mono(Boc)-protected 1,12-diaminododecane (**11**) [15] was performed. A modified procedure for mono-Boc-protection of the diamine **4** using 0.4 equiv. of di-*tert*-butyl pyrocarbonate (Boc_2O) in methanol–dichloromethane (3:2) was employed. VLC purification gave **11** in 78% yield. The conversion of **11** via **12** and **13** to **3** proceeded in 74% overall yield.

3. Conclusion

In conclusion, the present work represents an unprecedently high-yielding and cost-efficient route to philanthotoxin analogues; e.g. the overall yield of PhTX-12 (**3**) from diamine **4** was 58%. Although the selective protection of spermine (**1**) only afforded 43% of the desired **7**, this is reasonably close to the expected ca. 50%. This lower yield is due to the simultaneous formation of bis-Tfa-spermine protected at both primary amino groups, which ultimately results in the formation of di-Boc-spermine as a substantial by-product (18%). This by-product is, however, useful for solid-phase synthesis of philanthotoxins [6], or it may be converted to **7**. The method is very atom-economical, as most reactions are performed using ca. equimolar amounts of reactants. Also, the present procedure is based on readily available protected amino acid building blocks, and we have already prepared several other analogues with equally satisfactory results. An extensive biological testing of these analogues are currently being pursued, and these results will be published in due course. The present method can be expanded to analogues containing other diamine or polyamine moieties, the only requirement being the access to suitably protected precursors, possibly by extension of the procedures described for **7** or **11**. We believe that due to its sequential nature, the present method will be very convenient for parallel synthesis of larger amounts of members of small, focused combinatorial libraries, exhibiting variations in the amino acid and acyl moieties. The use of reverse-phase VLC is a major improvement with respect to the resources spent on purification. By using a manually packed VLC column of an appropriate size, it has proved possible to transfer elution results from analytical reverse-phase TLC (RP-18, R_f ca. 0.15–0.2) directly to the VLC column, thus

enabling gram-scale purification within a few hours with a gradient elution whenever necessary.

4. Experimental

4.1. Tri-Boc-spermine (7)

Spermine (**1**, 6.03 g, 30 mmol) was dissolved in MeOH (350 mL) under N_2. The mixture was cooled to −50 °C (±2 °C, manually temperature-controlled dry ice–acetone bath), when ethyl trifluoroacetate (4.1 mL, 35 mmol) was added dropwise during 0.5 h. The temperature was then allowed to rise to 0 °C, and the mixture was stirred for an additional 0.5 h at 0 °C. A solution of di-*tert*-butyl dicarbonate (30.5 g, 410 mmol) in MeOH (30 mL) was then added during 10 min. The reaction mixture was stirred at room temperature (r.t.) for 16 h (TLC showed a fully acylated product at R_f 0.8 in EtOAc), at which point H_2O (40 mL) and concentrated aqueous NaOH (30.6 mL) were added. The mixture was stirred at r.t. for 6 h followed by 16 h at 4 °C. Most of the MeOH was removed in vacuo. To the residual suspension was added CH_2Cl_2 (250 mL) and H_2O (100 mL). The aqueous layer was extracted repeatedly with CH_2Cl_2 (7 × 100 mL). The combined organic phases were dried (Na_2SO_4) and concentrated. The crude **7** (14.74 g) was dissolved in CH_2Cl_2 and loaded onto a VLC column (7 × 7 cm; Merck 60H silica gel), which was eluted successively with petroleum ether, CH_2Cl_2, CH_2Cl_2–MeOH (10:1), and CH_2Cl_2–MeOH–32% aqueous NH_3 (100:5:1 to 40:10:1). This yielded fraction A followed by **7** (4.49 g, R_f 0.54 in CH_2Cl_2–MeOH–32% aqueous NH_3; 30%) and di-Boc-spermine (2.21 g, R_f 0.11 in CH_2Cl_2–MeOH–32% aqueous NH_3; 18%). Repeated chromatography of fraction A afforded tetra-Boc-spermine (5.36 g, R_f 0.86 in CH_2Cl_2–MeOH–32% aqueous NH_3; 35%) and an additional amount of **7** (1.96 g, 13%).

4.2. Pentafluorophenyl butyrate

Pentafluorophenol (3.94 g, 21.4 mmol) and butyryl chloride (2.3 mL, 22.5 mmol) were dissolved in CH_2Cl_2 (100 mL). The mixture was cooled to 0 °C, and then pyridine (2.7 mL, 34.2 mmol) was added slowly. After the mixture was stirred at r.t. for 2 h, it was diluted with CH_2Cl_2 (250 mL), and subsequently washed with 0.1 M aqueous HCl (2 × 200 mL) and brine (200 mL). The organic phase was dried (Na_2SO_4), filtered, and concentrated in vacuo (bath temperature below 30 °C) to give the pure product as a liquid (5.31 g, 98%, purity at least 99%), which can be stored as a crystalline solid in a freezer for several months; R_f 0.85 (petroleum ether–EtOAc 2:1). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.98 (3H, t, $J = 7.3$ Hz, H-4), 1.74 (2H, sextet, $J = 7.3$ Hz, H-3), 2.57 (2H, t, $J = 7.3$ Hz, H-2). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.3 (C-4), 17.4 (C-3), 34.1 (C-2), 124.2 (C-1′), 135.4–141.7 (C-2′–C-6′), 168.5 (C-1). Anal. $C_{10}H_7O_2F_5$ requires C, 47.26; H, 2.78, Found: C, 47.54; H, 2.75%.

4.3. Mono-Boc-1,12-diaminododecane (11)

1,12-Diaminododecane (3.00 g, 15 mmol) was dissolved in CH_2Cl_2–MeOH (3:2, 150 mL), and then Boc_2O (1.31 g, 0.4 × 15 mmol) in MeOH (5 mL) was added. The mixture was stirred for 2 days. Concentration yielded a residue, which was suspended in CH_2Cl_2–MeOH (12.5:1, 54 mL) and loaded onto a VLC column (6 × 7 cm; Merck 60H silica gel). Gradient elution with hexane, CH_2Cl_2, CH_2Cl_2–MeOH (100:5), CH_2Cl_2–MeOH–32% aqueous NH_3 (200:10:1 to 100:10:1) yielded **11** (1.41 g, 78%).

4.4. PhTX-343 (2)

Tri-Boc-spermine (**7**, 2.0 g, 3.98 mmol) and Fmoc-Tyr(Bu^t)-OPfp (2.74 g, 1.1 × 3.98 mmol) were dissolved in dry CH_2Cl_2 (10 mL), and then Et_3N (0.55 mL, 3.97 mmol) was added. After the mixture was stirred at r.t. for 20 h (TLC with petroleum ether–EtOAc 1:1 showed product at R_f 0.3), the reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with water (2 × 50 mL). The organic layer was dried (Na_2SO_4) and concentrated. The residue was dissolved in dry THF (15 mL) and then octanethiol (39.8 mL, 10 × 3.98 mmol) and DBU (0.79 mL, 0.2 × 3.98 mmol) were added. The mixture was kept at r.t. for 24 h (after 3 h, TLC with CH_2Cl_2–MeOH–32% aqueous NH_3 200:10:1 showed almost full conversion; product at R_f 0.25). The reaction mixture was concentrated (removal of THF) and then loaded onto a VLC column (6 × 6 cm; Merck 60H silica gel), which was eluted successively with CH_2Cl_2 (300 mL) and CH_2Cl_2–MeOH–32% aqueous NH_3 200:10:0.5 (1500 mL) to give **9** (2.60 g, 91%).

A portion of **9** (2.09 g, 2.89 mmol) was treated with $C_3H_7COOPfp$ (1.48 g, 2.0 × 2.89 mmol) in the presence of Et_3N (0.55 mL, 1.37 × 2.89 mmol) for 2 h, when the mixture was concentrated. The residue was redissolved in CH_2Cl_2 (5 mL) and treated with an additional amount of $C_3H_7COOPfp$ (0.27 g, 0.37 × 2.89 mmol) for an additional 1 h. The reaction was followed by TLC with petroleum ether–acetone 2:1, R_f 0.21 and 0.32 correspond to the reactant and product, respectively. The reaction mixture was concentrated, and the residue was dissolved in CH_2Cl_2 (15 mL) and loaded onto a VLC column (6.5 × 6 cm; Merck 60H silica gel), which was eluted successively with hexane (300 mL), hexane–EtOAc (1:1, 500 mL), hexane–EtOAc (1:1.5, 1000 mL), and hexane–EtOAc (1:2, 2300 mL) to give **10** (1.95 g, 86%).

Deprotection of **10** (1.66 g, 2.10 mmol) was performed in CH_2Cl_2–TFA (9:1, 160 mL) at r.t. during 2.5 h. The mixture was concentrated to dryness, the residue was dissolved in 0.1% aqueous TFA (10 mL), and the solution was loaded onto a VLC column packed with Merck Lichroprep RP-18 (40–63 μm; 10 × 7 cm column), which was eluted with 0.1% aqueous TFA (500 mL), 1% MeCN (in 0.1% aqueous TFA; 1000 mL), 5% MeCN (in 0.1% aqueous TFA; 1000 mL), and 10% MeCN (in 0.1% aqueous TFA; 1500 mL). RP-18 TLC with 20% MeCN (in 0.1% aqueous TFA) showed **2** at R_f 0.38 in fractions eluted with 5–10% MeCN (in 0.1% aqueous TFA). Concentration of these fractions yielded **2** (1.63 g, 100%; 78% from **7**), ^{1}H- and ^{13}C-NMR spectra of which were identical with those of an authentic sample [6].

4.5. PhTX-12 (3)

Mono-Boc-1,12-diaminododecane (**11**, 0.89 g, 2.96 mmol) and Fmoc-Tyr(Bu^t)-OPfp (2.74 g, 1.1 × 2.96 mmol) were dissolved in dry CH_2Cl_2 (15 mL), and then Et_3N (0.41 mL, 2.96 mmol) was added. After the mixture was stirred at r.t. for 1 h, a distinct precipitation occurred, and more of dry CH_2Cl_2 (7 mL) was added. After an additional 1 h, when TLC with petroleum ether–EtOAc 2:1, showed a single spot with R_f 0.4, the reaction mixture was filtered extracting the insolubles with CH_2Cl_2–hexane (1:1, 50 mL). The filtrate was concentrated to give a crude product (1.99 g, 92%), a portion of which (1.89 g, 2.59 mmol) was dissolved in dry THF (20 mL), and then octanethiol (25.9 mL, 10 × 2.59 mmol) and DBU (0.52 mL, 0.2 × 2.59 mmol) were added. After 3 h at r.t. (TLC with CH_2Cl_2–MeOH–32% aqueous NH_3 200:10:1 showed the product at R_f 0.75) the reaction mixture was concentrated, and then loaded onto a VLC column (6 × 6 cm; Merck 60H silica gel), which was eluted successively with petroleum ether (400 mL), petroleum ether–EtOAc (1:1, 800 mL; and 1:2, 750 mL), and CH_2Cl_2–MeOH–32% aqueous NH_3 100:10:0.5 (1000 mL) to give **12** (1.23 g, 91%).

Acylation of **12** (1.21 g, 2.33 mmol) with $C_3H_7COOPfp$ (0.71 g, 1.2 × 2.33 mmol) in dry CH_2Cl_2 (10 mL) was performed in the presence of Et_3N (0.32 mL, 2.33 mmol) for 20 h (TLC with petroleum ether–ME_2CO 2:1 showed the product at R_f 0.32), when the mixture was concentrated. The residue was redissolved in CH_2Cl_2 (10 mL) and loaded onto a VLC column (6 × 6 cm; Merck 60H silica gel), which was eluted successively with hexane (500 mL) and hexane–EtOAc (1:1, 800 mL), which afforded **13** (1.32 g, 96%). Deprotection of **13** (1.17 g, 1.98 mmol) was performed in CH_2Cl_2–TFA (9:1, 10 mL) at r.t. for 3 h (RP-18 TLC with 60% MeCN–0.1% aqueous TFA showed the product at R_f 0.55). The mixture was concentrated to dryness, and the residue was dissolved in 50% MeCN–0.1% aqueous TFA (10 mL) and loaded onto a VLC column packed with Merck Lichroprep RP-18 (40–63 μm; 10 × 5 cm column), which was eluted with 5% MeCN (in 0.1% aqueous TFA; 200 mL), 10% MeCN (in 0.1% aqueous TFA; 500 mL), 20% MeCN (in 0.1% aqueous TFA; 500 mL), and 30% MeCN (in 0.1% aqueous TFA; 1000 mL). RP-18 TLC with 40% MeCN (in 0.1% aqueous TFA) showed **3** at R_f 0.30 in fractions eluted with 30% MeCN (in 0.1% aqueous TFA). Concentration of these fractions yielded **3** (0.93 g, 88%; 74% from **11**), ^{1}H- and ^{13}C-NMR spectra of which were identical with those of an authentic sample [6].

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