Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron Letters
Tetrahedron Letters 46 (2005) 6149–6153

Synthesis of labeled oligonucleotides through a new chemically cleavable linker

S. Mahajan,[a] S. Patnaik,[a] R. P. Gandhi,[b] K. C. Gupta[a,*] and P. Kumar[a,*]

[a]*Nucleic Acids Research Laboratory, Institute of Genomics and Integrative Biology, Mall Road, Delhi University Campus, Delhi 110 007, India*
[b]*Dr. B. R. Ambedkar Centre for Biomedical Research, University of Delhi, Delhi 110 007, India*

Received 14 February 2005; revised 22 June 2005; accepted 29 June 2005

Abstract—A synthesis of labeled oligonucleotides incorporating a new chemically cleavable linker **(III)** via a two-step method is described. The labeled oligomers obtained after cleavage and deprotection reactions [treatment with anhydrous *tert*-butylamine and dry methanol, 1:1 (v/v) for 12 h at room temperature, and lyophilization followed by subsequent reaction with aq NH_4OH and methylamine (40%), 1:1 (v/v) for 5 min at 65 °C] were analyzed by RP-HPLC. A distinctive feature of this protocol is that free oligomers can be recovered from their labeled analogs under mild conditions (0.2 M NaOH containing 0.5 M NaCl over 30 min at room temperature) and are comparable to the corresponding standard oligonucleotides (HPLC).

The introduction of reporter groups during solid phase synthesis of oligonucleotides has attracted attention over the past two decades. In response to hazards associated with the use and disposal of radionuclides, non-isotopic reporter groups have been increasingly utilized for detection of synthetic oligonucleotides.[1] Labeled oligonucleotides and nucleic acids have found various applications in detection of amplification products of polymerase chain reactions,[2] solid phase DNA sequencing,[3] hybridization probes,[4–7] etc. Conventional approaches for the synthesis of labeled synthetic oligonucleotides involve introduction of a nucleophilic functional group during the synthetic scheme and reaction with an appropriate electrophilic reporter moiety. Also, a few reporter phosphoramidite reagents and engineered supports exist, which allow one to label either the 5′- or 3′-end. The literature reports a few methods for permanent attachment of reporter moieties to oligonucleotides,[8–13] albeit, in these protocols, the linker and/or the label cannot be selectively removed to generate the free oligonucleotides from their labeled analogs. We reasoned that a cleavable linker would be useful for manipulation, purification or analysis of chemically synthesized oligonucleotides. A report in this direction relates to incorporation of, for example, a disulfide linkage into a linker between biotin and an oligonucleotide which is then cleaved by a reducing agent such as DTT.[14] However, the method is associated with the limitation that, during cleavage of the label from the oligomer, part of the linker is left attached to the oligonucleotide, thus returning a modified oligonucleotide. In Rothschild's method, formation of a thymine–thymine photodimer under UV-irradiation is a point of concern.[15,16] Fang and Bergstrom[17,18] have reported two fluoride cleavable biotinylating phosphoramidites for 5′-labeling, which can be further used for affinity purification of oligonucleotides. These methods suffer from the disadvantage of base specific multi-step synthesis, which further limits the strategy to a single reporter group (biotin). Another method from the same group results in 5′-phosphorylated DNA upon fluoride treatment.[18]

In view of the apparent limitations of the existing methods, we now report a new method for 5′-labeling of oligonucleotides employing commercially available reporter phosphoramidites, which are not base specific. This is based on a 5′-*O*-(4,4′-dimethoxytrityl)-3′(2′)-*O*-benzoyl-2′(3′)-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl)uridine phosphoramidite which was synthesized, and subsequently attached at the 5′-terminus of the growing

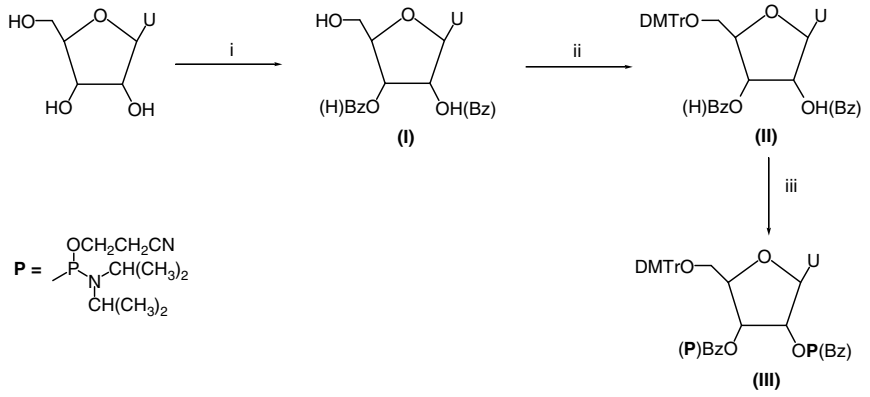
* Corresponding authors. Tel.: +91 11 27662491; fax: +91 11 27667471; e-mail addresses: kcgupta@igib.res.in; pkumar@igib.res.in

0040-4039/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2005.06.164

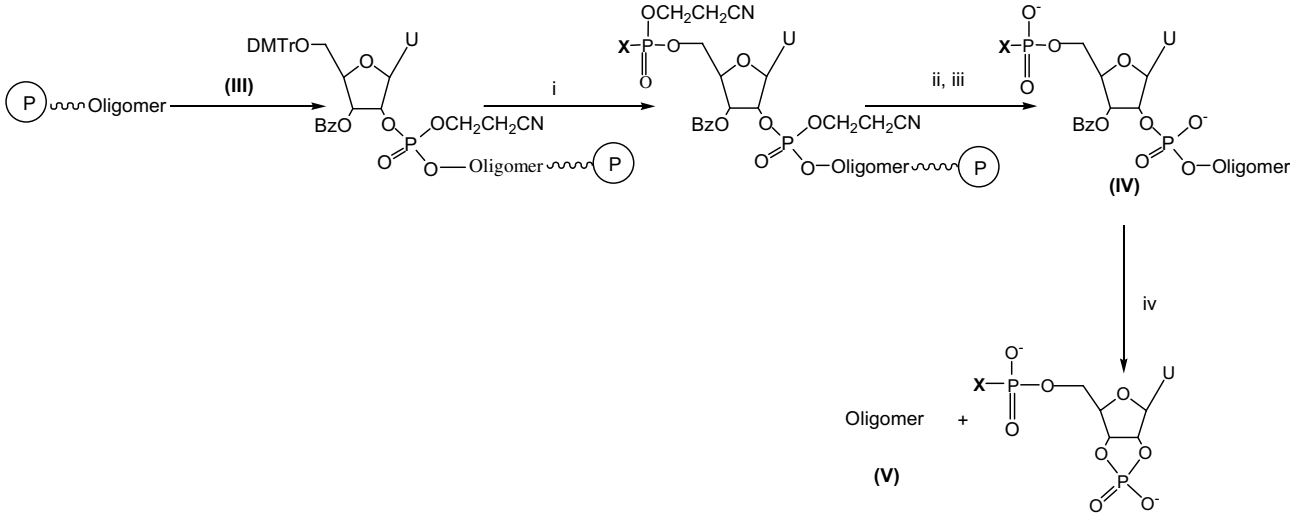
oligonucleotide chain, followed by coupling with the desired reporter phosphoramidite (Biotin, HEX, FAM phosphoramidite, etc.). The oligonucleotide sequence was then deprotected by treatment with anhydrous *tert*-butylamine and dry methanol, 1:1 (v/v) for 12 h at room temperature, then lyophilized and treated with aq NH_4OH and methylamine (40%), 1:1 (v/v) for 5 min at 65 °C to yield the labeled oligonucleotide.[19] The usefulness of the linker was demonstrated effectively by subjecting the labeled oligomer to the mild cleavage conditions reported earlier from our lab,[20] to afford the label-free analog whose authenticity was ascertained by HPLC.

The linker **(III)** was prepared from **(I)**[21] through reaction with DMTrCl at room temperature to give **(II)** (82% yield, characterized by IR, ^{1}H NMR and low-resolution MS[22]), which was phosphitylated using 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite to yield **(III)** in 89% yield (Scheme 1). The projected protocol for obtaining labeled and label-free oligonucleotides using linker **(III)** is outlined in Scheme 2.

While designing this strategy, we were encouraged from our earlier work on universal supports for oligonucleotide synthesis, where we reported fast deprotection conditions for the liberation of free oligomers from a *cis*-diol group bearing universal polymer supports.[20] We considered that introduction of linker **(III)** at the 5′-end of a polymer-bound oligomer, followed by coupling of the desired reporter phosphoramidite, would result in a labeled oligomer displaying cleavable properties. Apparent advantages of the above process are: (i) it enables the use of a reporter group of choice and (ii) it obviates the need to prepare a large number of phosphoramidite reagents of the reporter groups bearing cleavable linkers or the reporter group bearing nucleosides. To demonstrate the above protocol, we synthesized a number of oligonucleotides on a Pharmacia.LKB Gene assembler Plus.[23] The syntheses were performed on a



Scheme 1. Preparation of the cleavable universal phosphoramidite linker. Reagents and conditions: (i) (a) Bu_2SnO, reflux, MeOH; (b) BzCl, TEA, rt, 10 min; (ii) DMTrCl, py, rt, 24 h; (iii) 2-Cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite, tetrazole, MeCN.



Scheme 2. Methodology for the synthesis of labeled- and label-free oligonucleotides. Reagents and conditions: (i) Reporter phospharamidite (**X** = FAM) and tetrazole; (ii) anhydrous *tert*-butylamine and dry methanol (1:1 v/v), 12 h, rt; (iii) aq NH_4OH–$MeNH_2$ (40%) (1:1 v/v), 5 min, 65 °C; (iv) aq 0.2 N NaOH containing 0.5 M NaCl, 30 min, rt.

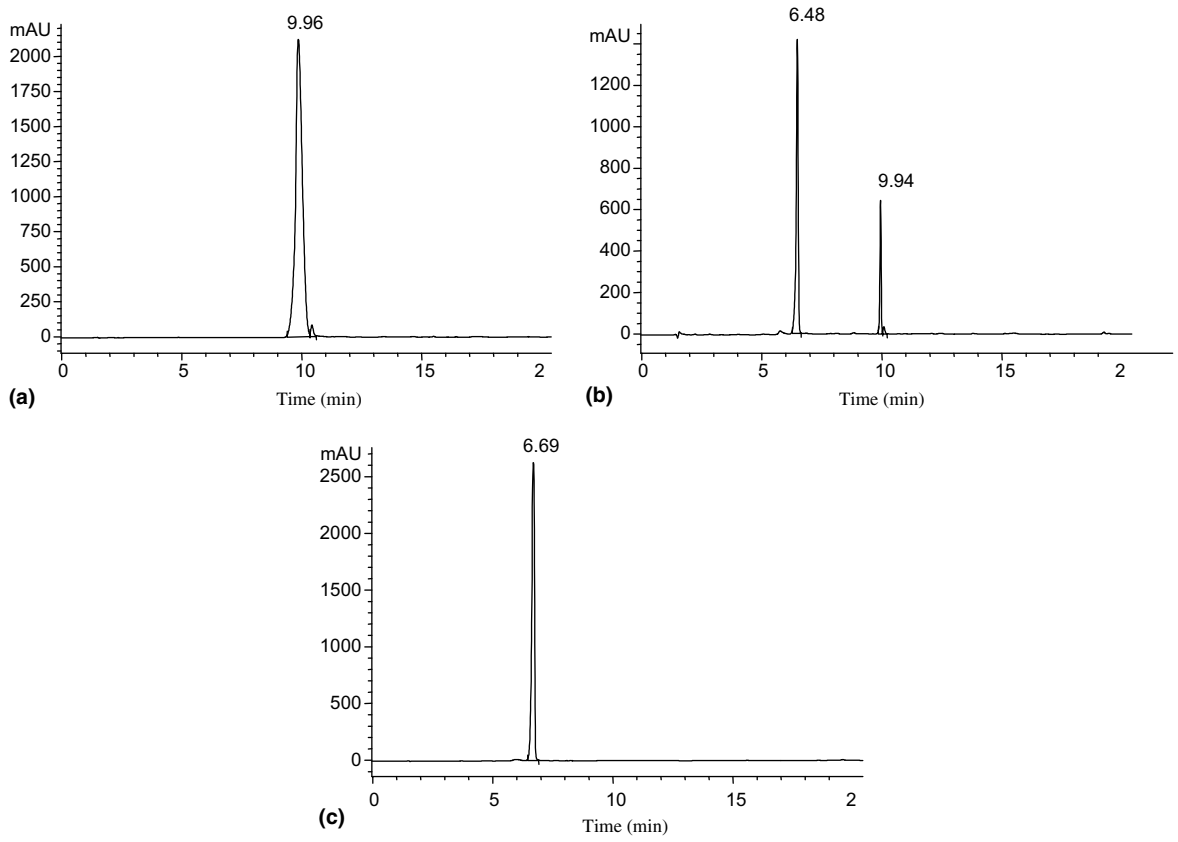


Figure 1. RP-HPLC profiles of (a) crude oligomer, FAM-U-dT; (b) co-injection of FAM-U-dT and standard dT; (c) co-injection of cleaved dT and standard dT.

0.2 μmol scale using labile nucleoside phosphoramidites (dA^{pac}, dG^{pac}, dC^{ac}). In order to examine the effect of fast deprotection conditions on the cleavage of labeled oligomers, a model experiment was designed in which the last coupling was performed with universal linker **(III)** on a standard dT support followed by coupling with the reporter phosphoramidite (X = FAM) with an extended coupling time (5 min). It was then subjected to the above deprotection conditions followed by analysis by reverse phase HPLC. The labeled dT analog's elution profile is depicted in Figure 1a. The identity of the eluent was established by co-injection with dT, as shown in Figure 1b. The peak with the retention time, $t_R = 9.94$ was found to be the desired labeled oligomer which eluted in almost quantitative yield.

For tethering FAM at the 5′-terminal of the synthesized oligomer, an oligomer d(CTA GTA CTA CTA CTA) was assembled on a standard dA support on 0.2 μmol scale using labile protecting groups. Labeling of the oligomer was accomplished in two steps using an automated DNA synthesizer,[23] by coupling the universal linker **(III)** to the polymer support-bound oligomer followed by coupling of the desired reporter phosphoramidite (X = FAM). Cleavage of the labeled oligomer from the support and removal of the various protecting groups (from nucleic bases and internucleotidic phosphates) were affected by treatment with anhydrous *tert*-butylamine and dry methanol, 1:1 (v/v) for 12 h at room temperature followed by aq NH_4OH and methylamine (40%), 1:1 (v/v), 65 °C, 5 min in a standard experiment. After usual work up and desalting, the labeled oligomer was concentrated using a Speed Vac and subjected to HPLC purification then lyophilized. The purified material was redissolved in water, divided into two portions and lyophilized again. One portion was dissolved in 0.1 M ammonium acetate buffer, pH 7.1 and analyzed on reversed phase HPLC.[24] The elution profile of the purified oligomer **(IV)** is shown in Figure 2a. The identity of oligomer **(IV)** was ascertained by co-injection with the standard oligomer d(CTA GTA CTA CTA CTA), whose elution pattern is shown in Figure 2b. The second portion of oligomer **(IV)**, on treatment with 0.2 M NaOH containing 0.5 M NaCl (200 μL) for 30 min at rt, led to cleavage of the universal linker from **(IV)**, quantitatively[20] (Scheme 2). The resulting solution, after neutralization with acetic acid, was concentrated in a Speed Vac, redissolved in 0.1 M ammonium acetate buffer and analyzed by HPLC. Again, the free oligomer **(V)** [Fig. 2c] eluted with a lower retention time as compared to the labeled oligomer **(IV)**. The identity of the released oligonucleotide was confirmed by co-injection with the standard oligomer, d(CTA GTA CTA CTA CTA), its elution pattern is shown in Figure 2d. In an analogous manner, Biotin and HEX-phosphoramidite were attached to the oligomer sequence bound to the polymer support after coupling to the universal linker **(III)**.

In conclusion, a universal linker based on a uridine nucleoside has been designed and synthesized, which

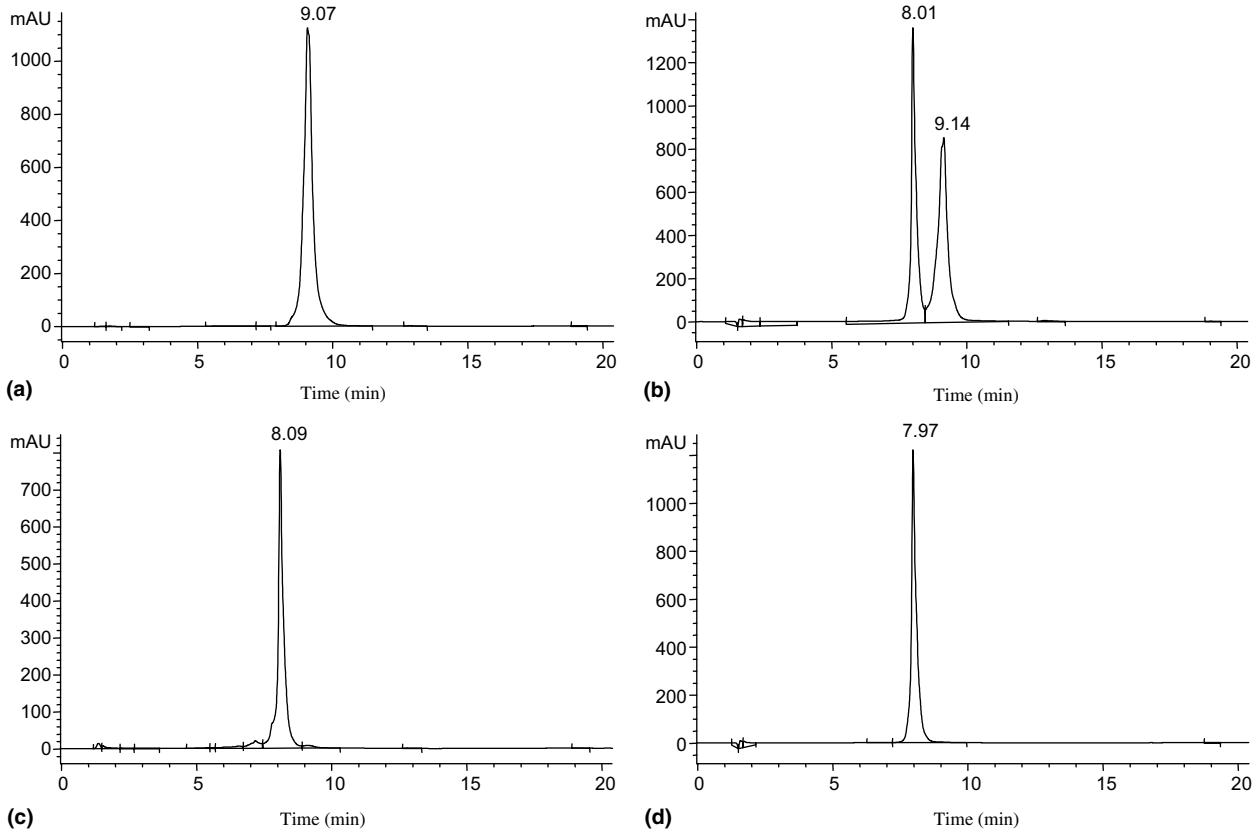


Figure 2. RP-HPLC profiles of (a) oligomer, FAM-U-d(CTA GTA CTA CTA CTA) **(IV)**; (b) co-injection of FAM-U-d(CTA GTA CTA CTA CTA) **(IV)** and the standard d(CTA GTA CTA CTA CTA); (c) d(CTA GTA CTA CTA CTA) **(V)** obtained after treatment with 0.2 M NaOH containing 0.5 M NaCl for 30 min at rt; (d) co-injection of d(CTA GTA CTA CTA CTA) **(V)** with the standard d(CTA GTA CTA CTA CTA). 'U' denotes chemically cleavable linker **(III)**.

can be used to incorporate cleavable properties into oligonucleotides.

Acknowledgements

Financial support from CSIR Task Force Project (NNI-OSB, COR010) is gratefully acknowledged. S.M. thanks the Council of Scientific and Industrial Research (CSIR), New Delhi, India, for the award of a Senior Research Fellowship.

References and notes

1. Beck, S.; O'Keeffe, T.; Coull, J. M.; Koster, H. *Nucleic Acid Res.* **1989**, *17*, 5115–5123.
2. Green, A.; Roopra, A.; Vaudin, M. *Nucleic Acid Res.* **1990**, *18*, 6163–6164.
3. Hultman, T.; Stahl, S.; Hornes, E.; Uhlen, M. *Nucleic Acid Res.* **1989**, *17*, 4937–4946.
4. De Vos, M.-J.; Cravador, A.; Lenders, J.-P.; Houard, S.; Bollen, A. *Nucleosides Nucleotides* **1990**, *9*, 259–273.
5. Bengtstrom, M.; Harju, L.; Syvanen, A.-C. *Nucleosides Nucleotides* **1991**, *10*, 507–509.
6. Reyes-Engel, A.; Dieguez-Lucena, J. L. *Nucleic Acid Res.* **1993**, *21*, 759–760.
7. Niemeyer, C. M.; Sano, T.; Smith, C. L.; Cantor, C. R. *Nucleic Acid Res.* **1994**, *22*, 5530–5539.
8. Misiura, K.; Durrant, I.; Evans, M. R.; Gait, M. J. *Nucleic Acid Res.* **1990**, *18*, 4345–4354.
9. Kumar, P.; Sharma, A. K.; Gupta, K. C. *Nucleosides Nucleotides* **1996**, *15*, 1263–1273.
10. Kumar, P.; Bhatia, D.; Garg, B. S.; Gupta, K. C. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1761–1766.
11. Manoharan, M.; Inamati, G.; Tivel, K. L. *Nucleosides Nucleotides* **1997**, *16*, 1411–1413.
12. Pieles, U.; Sproat, B. S.; Lamm, G. M. *Nucleic Acid Res.* **1990**, *18*, 4355–4360.
13. Zhao, Z.; Ackroyd, J. *Nucleosides Nucleotides* **1999**, *18*, 1231–1234.
14. Shimkus, M.; Levy, J.; Herman, T. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2593–2597.
15. Olejnik, J.; Sonar, S.; Krzymanska-Olejnik, E.; Rothschild, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7590–7594.
16. Olejnik, J.; Krzymanska-Olejnik, E.; Rothschild, K. J. *Nucleic Acid Res.* **1996**, *24*, 361–366.
17. Fang, S.; Bergstrom, D. E. *Nucleic Acid Res.* **2003**, *31*, 708–715.
18. Fang, S.; Bergstrom, D. E. *Bioconjug. Chem.* **2003**, *14*, 80–85.
19. Reddy, M. P.; Hanna, N. B.; Farooqui, F. *Tetrahedron Lett.* **1994**, *35*, 4311–4314.
20. Kumar, P.; Gupta, K. C. *Nucleic Acid Res.* **1999**, *27*, e2.
21. Wagner, D.; Verheyden, J. P. H.; Moffatt, J. G. *J. Org. Chem.* **1974**, *39*, 24–30.
22. Characterization data, **II**: IR (thin film) ν (cm^{-1}) = 1033, 1255, 1682, 1702, 3049, 3502. ^1H NMR (300 MHz,

$CDCl_3$): δ (ppm): 2.16 (s, 1H, $C_2'OH$), 3.72–3.79 (m, 9H, $2 \times OCH_3$, $C_5'H_2$), 3.99 (m, 1H, C_4'), 4.09 (m, 1H, C_3'), 4.66 (m, 1H, $C_2'H$), 5.65 (m, 1H, $C_1'H$), 6.21 (d, $J = 8$ Hz, 1H, C_5 H), 6.70–7.60 (m, 18H, Ar), 8.05 (d, $J = 6$ Hz, 1H, C_6 H). Low resolution mass: 673.34 ($M+Na^+$).

23. Gene Assembler Plus Manual, Uppsala, Sweden, 1988.
24. Analytical HPLC was performed on an Agilent 1100 series fitted with a diode array detector set at 254 nm using a Hypersil Gold RP-18 column (125 mm × 4 mm). Solvent A = 0.1 M ammonium acetate, pH 7.1 and solvent B = 100% MeCN; Gradient: 0–45% B in 35 min; Flow rate: 1.0 mL/min.