ELSEVIER

Journal of Chromatography A, 878 (2000) 165–170

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Large degree of racemization observed in the amide bond forming reaction on silica gel

Anle Yang, Andrew P. Gehring, Tingyu Li*

Department of Chemistry, Box 1822-B, Vanderbilt University, Nashville, TN 37235, USA

Received 17 September 1999; received in revised form 25 February 2000; accepted 7 March 2000

Abstract

A systematic study of racemization from the coupling of DNB-L-Leu and 3-aminopropyl silica gel with several amide coupling reagents was investigated. Significant amounts of racemization were observed from all except one coupling reagent. In comparison, similar reactions completed in homogeneous solution can be accomplished with much lower racemization and in much higher yields.

Keywords: Racemization; Chiral stationary phases, LC; Enantiomer separation; Amides

1. Introduction

One of the popular methods to immobilize stationary phases onto silica gel involves amide bond formation (for some examples, see Ref. [1]). In this approach, stationary phases derivatized with a free carboxylic acid group are coupled to silica gel derivatized with an amino group, typically 3-aminopropylsilica gel, by means of a proper coupling reagent. The advantage of this method is that stationary phases with a free carboxylic acid group, as well as silica gel derivatized with an amino group, are stable and often easily prepared. Problems often encountered with this immobilization method, however, include relatively low coupling yields and possible racemization in the case of chiral stationary phases. Since this reaction is commonly used in stationary phase preparations, we decided to study the yield and possible racemization in this coupling reaction with various amide coupling reagents systematically. In this paper, we would like to report the large degree of racemization observed in this amide bond forming reaction on silica gel.

It should be pointed out that studies regarding the coupling yields may have been performed previously. However, no such studies have been published to the best of our knowledge. The racemization in these peptide coupling reactions may have also been investigated indirectly. However, the reliable determination of the degree of racemization on silica gel was not possible prior to our oxidative cleavage method which was published recently [2,3].

2. Experimental

2.1. General supplies and equipment

N-(3,5-Dinitrobenzoyl)-L-leucine (DNB-L-Leu),

*Corresponding author. Tel./fax: +1-615-3438-466.
E-mail address: tingyu.li@vanderbilt.edu (T. Li)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00332-0

all other chemicals, and solvents were purchased from either Aldrich, Fluka, or Fisher Scientific unless otherwise noted. HPLC-grade Allsphere silica gel (particle size 5 μm, pore size 80 Å, and surface area 220 m^2/g) was purchased from Alltech (Deerfield, IL, USA). Selecto silica gel (32–63 μm) from Fisher Scientific was used for flash column chromatographic purification of target compounds. Thin-layer chromatography (TLC) was completed using EM silica gel 60 F-254 TLC plates (0.25 mm). A S-N1N-Naphthylleucine column was purchased from Regis Technologies (Morton Grove, IL, USA). Elemental analyses were conducted by Atlantic Microlab, Norcross, GA, USA. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded with a Bruker 300 MHz instrument. HPLC analyses were carried out on a Beckman analytical gradient system (System Gold).

2.2. Reagent abbreviations

NMM: *N*-methylmorpholine; PyBop: benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate; Fmoc: 9-fluorenylmethoxycarbonyl; DIC: diisopropylcarbodiimide; HOAt: 1-hydroxy-7-azabenzotriazole; HOBt: 1-hydroxybenzotriazole; DPPA: diphenylphosphoryl azide; EEDQ: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; FDPP: Pentafluorophenyl diphenylphosphinate; HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DNB: 3,5-dinitrobenzoyl.

2.3. Synthesis of DNB-Leu-silica gel (**2**) using different coupling reagents

Aminopropyl silica gel (prepared according to Pirkle et al. [4]) (100 mg) and DNB-L-Leu (2.0 equiv. to the aminopropyl group on silica gel) were combined in a 1-dram screwcap vial. In a second 1-dram vial, coupling reagents and base (as needed) were combined and dissolved in dimethyl formate (DMF)–CH_2Cl_2 (1:9, 0.5 ml) with sonication for 5 min. This mixture was transferred by Pasteur pipette to the vial containing the other reactants. The resulting reaction mixture was stirred at room temperature for 10 h. The product was then vacuum-filtered and washed with 10% trifluoroacetic acid (TFA)–CH_2Cl_2, MeOH, and CH_2Cl_2 until residual DNB-L-Leu was no longer present by TLC. The surface coverage of the resulting stationary phases were determined by elemental analysis as described previously [2,3].

Amounts of coupling reagents relative to the amount of 3-aminopropyl group on silica gel: DIC–HOBt: DIC 2.4 equiv., HOBt 2.4 equiv.; DIC–HOAt: DIC, 2.4 equiv., HOAt 2.4 equiv.; DPPA: DPPA 2.4 equiv., NMM, 3.0 equiv.; EEDQ: EEDQ 2.4 equiv.; FDPP: FDPP 2.4 equiv., NMM 3.0 equiv.; HATU: HATU 2.4 equiv., NMM 3.0 equiv.; PyBop: PyBop 2.4 equiv., NMM 3.0 equiv.

2.4. Synthesis of DNB-Leu-$NH(CH_2)_3Si(OEt)_3$ (**3**) using different coupling reagents

To a mixture of 3-aminopropyltriethoxysilane (0.010 mmol) and DNB-L-Leu (6.5 mg, 0.020 mmol) in DMF–CH_2Cl_2 (1:9, 0.1 ml) were added the corresponding coupling reagents in DMF–CH_2Cl_2 (1:9, 0.2 ml). The resulting solution was stirred at room temperature for 2 h[1]. After that, solvents were removed in vacuo and the desired product **3** was isolated from the resulting residue by preparative TLC [mobile phase: EtOAc–CH_2Cl_2 (1:3), R_F= 0.68]. The enantiomeric purity of all resulting products was determined by the method described below (see Section 2.7). ^{1}H NMR ($CDCl_3$) of **3** as determined from the product isolated from EEDQ coupling: δ 0.65 (t, J=8.0 Hz, 2H), 0.95 (d, J=4.9 Hz, 6H), 1.15–1.30 (m, 9H), 1.6–1.8 (m, 5H), 3.3 (m, 2H), 3.8 (q, J=6.9 Hz, 6H), 4.65 (m, 1 H), 6.45 (m, 1H), 8.10 (m, 1H), 8.95 (d, J=2.0 Hz, 2H), 9.15 (t, J=2.0 Hz, 1H).

Amounts of coupling reagents relative to the amount of 3-aminopropyltriethoxysilane: DIC–HOBt: DIC 2.4 equiv., HOBt 2.4 equiv.; DIC–HOAt: DIC, 2.4 equiv., HOAt 2.4 equiv.; DPPA: DPPA 2.4 equiv., NMM, 3.0 equiv.; EEDQ: EEDQ 2.4 equiv.; FDPP: FDPP 2.4 equiv., NMM 3.0 equiv.; HATU: HATU 2.4 equiv., NMM 3.0 equiv.; PyBop: PyBop 2.4 equiv., NMM 3.0 equiv.

[1]With longer reaction time (12 h), the enantiomeric purity stayed the same. However, the isolated amounts of product were lower.

2.5. Synthesis of DNB-Leu-NHCH$_2$CH$_2$CH$_3$ (**4**) using different coupling reagents

To a mixture of *n*-propylamine (0.010 mmol) and DNB-L-Leu (6.5 mg, 0.020 mmol) in DMF–CH_2Cl_2 (1:9, 0.1 ml), were added the corresponding coupling reagents in DMF–CH_2Cl_2 (1:9, 0.2 ml). The resulting solution was stirred at room temperature for 10 h. After that, solvents were removed in vacuo and the desired product **4** was isolated from the resulting residue by preparative TLC [mobile phase: EtOAc–CH_2Cl_2 (1:3), R_F=0.85]. The yields and enantiomeric purity of the resulting products were determined by the methods described below (Sections 2.6 and 2.7). ^{1}H NMR ($CDCl_3$) of **4** as determined from the product isolated from EEDQ coupling: δ 0.90–1.00 (m, 9H), 1.55 (m, 2H), δ 1.6–1.9 (m, 3H), 3.13 (m, 1H), 3.32 (m, 1H), 4.72 (m, 1H), 6.61 (t, J=5.6 Hz, 2H), 8.83 (d, J=8.0 Hz, 1H), 8.96 (d, J=2.0 Hz, 2H), 9.08 (t, J=2.0 Hz, 1H).

Amounts of coupling reagents relative to the amount of *n*-propylamine: DIC–HOBt: DIC 2.4 equiv., HOBt 2.4 equiv.; DIC–HOAt: DIC, 2.4 equiv., HOAt 2.4 equiv.; DPPA: DPPA 2.4 equiv., NMM, 3.0 equiv.; EEDQ: EEDQ 2.4 equiv.; FDPP: FDPP 2.4 equiv., NMM 3.0 equiv.; HATU: HATU 2.4 equiv., NMM 3.0 equiv.; PyBop: PyBop 2.4 equiv., NMM 3.0 equiv.

2.6. Quantification of amide **4** by high-performance liquid chromatography (HPLC)

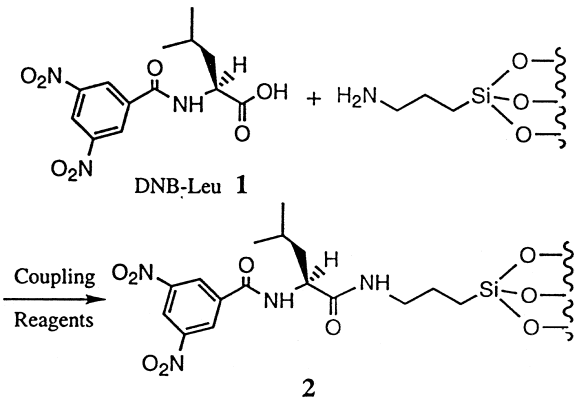
The amount of amide **4** generated from the coupling reaction of *n*-propylamine and DNB-L-Leu was determined by HPLC equipped with a UV detector using an S-N1N-Naphthylleucine column [mobile phase: 2-propanol–CH_2Cl_2 (60:40)]. The calibration was obtained by using the pure amide **4** synthesized with EEDQ coupling. The amount of amide **4** produced in a particular experiment was calculated based on the total peak areas of both the *R* and *S* forms of amide **4**.

2.7. Enantiomeric purity determination of **3**, **4**, and **5** (see Scheme 3 for compound **5**)

The enantiomeric purity of **3** and **4** was determined by HPLC using the S-N1N-Naphthylleucine column mentioned above [mobile phase: 2-propanol–CH_2Cl_2 (60:40)]. The enantiomeric purity of **5** was determined by HPLC using the same column with a different mobile phase [mobile phase: CH_3OH–CH_2Cl_2 (85:15)]. The enantiomeric excess of the starting material DNB-L-Leu was determined to be 96% (mobile phase: 85% isopropanol (IPA) in CH_2Cl_2) following a similar procedure.

3. Results and discussion

The system we chose to study was the coupling reaction between dinitrobenzoylleucine (DNB-L-Leu) and 3-aminpropyl silica gel (Scheme 1), a reaction originally used by Pirkle and Welch to prepare the chiral leucine column [5]. In our studies, seven amide coupling reagents were investigated (see Section 2.2 for coupling reagent abbreviations). Some of them are well known in stationary phase preparation, while others are relatively new developments in peptide synthesis. Among these amide coupling reagents, EEDQ was the reagent used by Pirkle for the preparation of the chiral leucine column. DIC [6] is similar to DCC in chemical structure and reactivity, but is easier to handle. HOBt is a standard additive used to minimize racemization in amide coupling reactions [6], while HOAt is reportedly an more effective additive for the same purpose [7]. Both HOBt and HOAt are often used in association with DCC or DIC coupling reagent. Another useful amide coupling reagent, DPPA, was initially de-

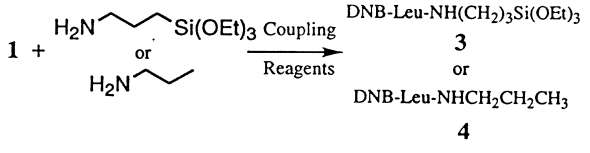


Scheme 1. Amide bond forming reaction on silica gel.

veloped by Shioiri for the Curtius reaction [8], while FDPP is a phosphinate based reagent, reportedly capable of effecting racemization-free amide coupling [9]. PyBop, a phosphonium salt, is a relatively new peptide coupling reagent capable of effecting a rapid amide bond forming reaction [10], and HATU, a uronium salt, is another relatively new coupling reagent also capable of fast amide bond formation [7]. Both HATU and PyBop are becoming increasingly popular in solid-phase peptide synthesis, as they can effect quick amide formation with little to no racemization. PyBop and HATU are very attractive candidates for amide bond forming reactions on silica gel due to their proven efficiency in solid-phase peptide synthesis.

These coupling reactions are usually carried out either at room temperature or at 0°C. DMF, CH_2Cl_2, or a mixture of DMF–CH_2Cl_2 are often the solvents of choice in these coupling reactions. Although for a specific coupling reaction (specific reagent and reactants), an optimal coupling solvent and temperature may exist. In our experience, the reaction outcome is usually not strongly dependant on the reaction temperature (0°C versus room temperature) or the choice of solvents (DMF versus mixture of DMF–CH_2Cl_2). Moreover, in order to compare the coupling efficiency of various coupling reagents, it is advantageous to keep the reaction conditions as close to each other as possible. Therefore, all the coupling reactions reported in this article were performed at room temperature using a mixture of DMF–CH_2Cl_2 (1:9) as the solvent.

For control experiments, we studied the coupling between DNB-L-Leu and 3-aminopropyltriethoxysilane, a good model for 3-aminopropylsilica gel (Scheme 2). The product was isolated by normal-phase TLC and its enantiomeric purity was analyzed by chiral HPLC using the S-N1N-Naphthylleucine column. The results obtained for this solution coupling follow expectations as inferred from various



Scheme 2. Amide bond formation in homogeneous solution.

Table 1
Racemization observed in the coupling of **1** with $NH_2(CH_2)_3Si(OEt)_3$, $NH_2(CH_2)_2CH_3$, and aminopropylsilica gel[a]

Coupling reagent	Base	D-Content (%)		
		2 (yield)	**3**	**4** (yield)
DIC–HOAt	None	33 (53%)	4.2	6.2 (82%)
DIC–HOBt	None	30 (55%)	11	12 (100%)
DPPA	NMM	34 (22%)	6.4	16 (78%)
EEDQ	None	3.7 (47%)	2.2	3.6 (88%)
FDPP	NMM	40 (10%)	11	18 (76%)
HATU	NMM	30 (45%)	5.0	3.4 (98%)
PyBop	NMM	40 (57%)	14	14 (100%)

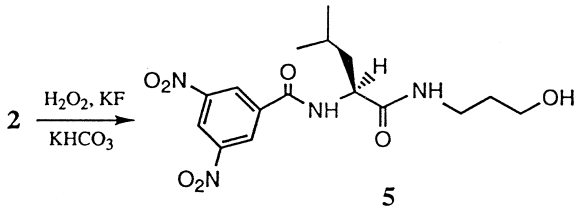
[a] Starting material (**1**) contains about 2% D-enantiomer. Coupling yields are in parentheses.

literature reports (Table 1). For example, DIC–HOAt, EEDQ and HATU could all effect the coupling of DNB-L-Leu with 3-aminopropyltriethoxysilane without inducing a significant amount of racemization, while DIC–HOBt and DPPA introduced more racemization. Somewhat surprising is that fact that FDPP and PyBop also introduced noticeable amounts of racemization.

Unfortunately, the coupling yields could not be determined reliably in this case due to product instability. However, the coupling between DNB-L-Leu and *n*-propylamine (Scheme 2), in which the expected product is stable, yielded the corresponding amide **4** in excellent yields with most of these coupling reagents. Moreover, similar degrees of racemization were obtained for this coupling reaction when compared with the coupling reaction involving 3-aminopropyltriethoxysilane. Therefore, from the solution data, HATU, EEDQ and DIC–HOAt all appear to be excellent amide bond forming reagents, capable of effecting coupling reactions in high yield and with low racemization.

On silica gel, however, the results are very different. First, none of the coupling reagents could provide high coupling yields. More importantly, significant amounts of racemization (the maximum D-content is 50%!) were observed with all except one coupling reagent (EEDQ). Interestingly, EEDQ was the reagent used by Pirkle for the preparation of chiral leucine and other stationary phases on silica gel [5].

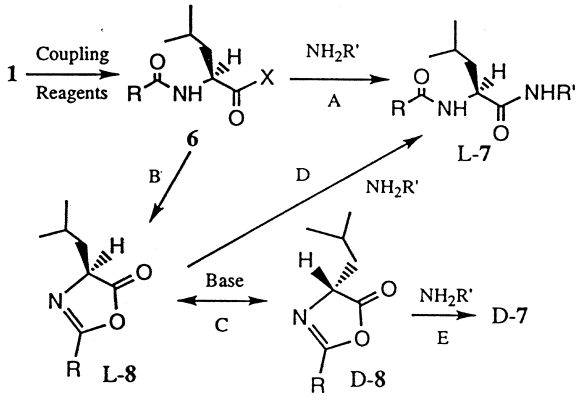
The yields reported in Table 1 for coupling reactions on silica gel were determined by elemental



Scheme 3. Oxidative cleavage of carbon–silica bond to determine the enantiomeric purity of chiral leucine stationary phases.

analysis, while enantiomeric purity of the stationary phases on silica gel was determined by the oxidative cleavage method reported previously (Scheme 3) [2].

The significant amount of racemization observed in the amide coupling reactions on silica gel is unexpected, considering that the same amount of racemization was not observed in solution. The mechanisms for racemization in amide bond formation in peptides are well studied, and the most likely racemization path is through the formation of an azlactone (Scheme 4) [11]. Once an amino acid is activated by a coupling reagent, two reaction paths become available. The activated amino acid (**6**) could react directly with the amine, leading to the formation of the desired product L-**7** without any racemization (path A). The other scenario involves an intramolecular rearrangement, leading to the formation of an azlactone (**8**) (path B). The α-proton in the azlactone (**8**) is rather acidic due to extensive conjugation. Therefore, azlactone (**8**) can be deprotonated and reprotonated, causing its racemiztion (path C). Both enantiomers of azlactone could react further



Scheme 4. Racemization through an azlactone intermediate (**8**). R=3,5-dinitrophenyl. X indicates an active intermediate.

with the amine, leading to their corresponding amides as the final products (paths D and E).

According to this mechanism, the extent of racemization in the product is determined by the relative rates of five reaction steps (A, B, C, D, E). If the reaction of the amine with the azlactone or the initially formed activated intermediate is fast (steps A and D) relative to steps B and C, low racemization is expected. If steps B and C are fast relative to steps A and D, a large degree of racemization will result.

Applying this analysis to our system, steps B and C should have similar reaction rates in both solution and on silica gel, as they both are determined by the structure of the acid **1** and the coupling reagents common to both conditions. Steps A, D, and E, however, can show different reaction rates. It is likely that when the amino group is attached to a solid support, its reaction with another compound could be impeded. Consequently, steps A and D become relatively slower and thus more racemization would be observed when the coupling reaction is performed on silica gel. The fact that no significant racemization was observed with EEDQ in both solution and on silica gel probably indicates that conversion from the activated amino acid to the azlactone is slow (step B) compared to its direct conversion to the final product (step A).

As pointed out by one reviewer, the kinetics of reactions on solid support has been compared with that of similar reactions in solution. While it is generally believed that solid-supported reactions tend to be slower than their counterparts in solution [12], recent evidence suggests solid-supported reactions can be faster than similar reactions in solution [13]. Therefore, the discussion described in the last paragraph, which attributes the significant amounts of racemization to the possibly slower reaction kinetics in the solid phase, may prove inadequate. At this time, however, we still believe that this is the most logical explanation.

As to the low coupling yields on silica gel, surface accessibility of aminopropyl groups could serve as one limiting factor. However, a similar experiment using controlled pore glass (pore size 350 Å) in which free access to the surface is normally assumed, yielded a slightly lower coupling yield and a similar degree of racemization (coupling reagents: DIC–HOAt, yield: 49%, and D: 36%). Therefore,

reasons that have not been successfully identified so far are more likely responsible for the low coupling yields.

Racemization in chiral stationary phase preparation is expected to lead to reduced separation factors. To investigate such an influence, two columns were packed, one from a stationary phase prepared from EEDQ coupling, and the other from DIC–HOBt coupling. Indeed, the stationary phase prepared from EEDQ coupling could resolve racemic 2,2,2-trifluoro-1-(9-anthryl)ethanol with a higher separation factor of 1.44 (5% IPA–hexanes) (dead time t_0 was measured with 1,3,5-tri-*tert*.-butylbenzene as the void volume marker according to Prikle and Welch [14]), while the column prepared from DIC–HOBt coupling had a separation factor of only 1.22 under the same conditions.

It should be noted that, this seemingly simple reaction is actually one of the more challenging reactions to accomplish without introducing any racemization. The electron withdrawing nature of the dinitrobenzoyl group increases the acidity of the α-proton in the azlactone (**8**) when compared with other amino acid derivatives, and increasing acidity in turn leads to faster racemization of the azlactone intermediate (step C).

4. Conclusions

Racemization resulting from the coupling of DNB-L-Leu and 3-aminopropyl silica gel with several amide coupling reagents was investigated with the oxidative cleavage method reported previously. Significant amounts of racemization were observed in all except one coupling reagent (EEDQ). In comparison, several reagents were able to effect the solution coupling of DNB-L-Leu with 3-aminopropyltriethoxysilane, a good model for 3-aminopropylsilica gel, without significant amounts of racemization. In solution, near quantitative coupling yields could be achieved with several coupling reagents. In contrast, all the reagents fail to provide high coupling yields on silica gel. These results indicate that the reaction on silica gel can be very different from an almost identical reaction in solution. Therefore, careful characterization of the final product is very important in the preparation of any stationary phases. To this end, the oxidative cleavage method proves useful in determining the enantiomeric purity of chiral stationary phases.

Acknowledgements

The financial support from Vanderbilt University, in the form of start-up fund, research council, and natural science fund is appreciated. The project is also supported in part with an ACS PRF grant (31692-G1).

References

[1] S.R. Perrin, W.H. Pirkle, in: S. Ahuja (Ed.), Chiral Separations by Liquid Chromatography, ACS, Washington, DC, 1991, pp. 43–66.
[2] A. Yang, T. Li, Anal. Chem. 70 (1998) 2827.
[3] For a review of other characterization methods, see: L.C. Sander, S.A. Wise, CRC Crit. Rev. Anal. Chem. 18 (1987) 332.
[4] W.H. Pirkle, D.W. House, J.N. Finn, J. Chromatogr. 192 (1980) 143.
[5] W.H. Pirkle, C.J. Welch, J. Org. Chem. 49 (1984) 138.
[6] M. Bodanszky, in: Principles of Peptide Synthesis, 2nd ed., Springer-Verlag, New York, 1993, pp. 38–47.
[7] L.A. Carpino, J. Am. Chem. Soc. 115 (1993) 4397.
[8] T. Shioiri, K. Ninomiya, S. Yamada, J. Am. Chem. Soc. 94 (1972) 6203.
[9] S. Chen, J. Xu, Tetrahedron Lett. 32 (1991) 6711.
[10] J. Coste, D. Le-Nguyen, B. Castro, Tetrahedron Lett. 31 (1990) 205.
[11] M. Bodanszky, in: Principles of Peptide Synthesis, 2nd ed., Springer-Verlag, New York, 1993, pp. 170–177.
[12] P. Hodge, in: D.C. Sherrington, P. Hodge (Eds.), Synthesis and Separations Using Functional Polymers, Wiley, New York, 1988, pp. 43–122.
[13] B. Yan, J.B. Fell, G. Kumaravel, J. Org. Chem. 61 (1996) 7467.
[14] W.H. Pirkle, C.J. Welch, J. Liq. Chromatogr. A 14 (1991) 1.