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Optimization of Solid-Phase Synthesis of [Ala^8]-dynorphin A[1–3]

Núria A. Solé and George Barany*

Department of Chemistry, University of Minnesota, Minneapolis, MN 55455

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[Ala^8]-dynorphin A, a potent κ-selective opioid heptadecapeptide with numerous sensitive side-chain residues, has been prepared by solid-phase synthesis using base-labile N^α-9-fluorenylmethyloxycarbonyl (Fmoc) protection and side-chain anchoring to a tris(alkoxy)benzylamide "PAL"-resin. Final cleavage and deprotection was carried out with reagent R, trifluoroacetic acid-thioanisole–1,2-ethanedithiol–anisole (90:5:3:2), reagent K, trifluoroacetic acid–phenol–water–thioanisole–1,2-ethanedithiol (82.5:5:5:5:2.5), and reagent B, trifluoroacetic acid–phenol–water–triisopropylsilane (88:5:5:2); optimal reaction and workup conditions are described. Crude peptide products were evaluated by analytical high-performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE), and direct fast atom bombardment and ion electrospray mass spectrometry (FABMS and ESMS). Tryptophan alkylation side reactions involving a 2,4,6-trimethoxybenzyl (Tmob) group from asparagine or 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc) groups from arginines occur under insufficiently long cleavage times and/or when certain scavenger components are omitted from the cleavage cocktails, but can be minimized under the best conditions. Conditions with reagent B, 1 h, 25 °C, and extractive workup, were followed up preparatively to provide [Ala^8]-dynorphin A in excellent purity (>99%) and 58% overall isolated yield based on the C-terminal amino acid anchored on the resin.

Methods for the solid-phase synthesis (SPPS) of peptides using the base-labile N^α-9-fluorenylmethyloxycarbonyl (Fmoc) protecting group have considerable appeal because they allow the final cleavage and deprotection step to be carried out under relatively mild orthogonal acidolytic conditions.[4–6] Compatible side-chain protection is provided by appropriate *tert*-butyl (*t*Bu), triphenylmethyl (trityl, Trt), 2,4,6-trimethoxybenzyl (Tmob), and 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc) derivatives, which in acid give rise to quite stable carbonium ions.[4–8] These ions can in turn alkylate residues as cysteine, methionine, tyrosine, and particularly tryptophan. Such side reactions are usually irreversible and can be mitigated but not necessarily eliminated by addition to the cleavage cocktail of one or more scavengers.[2,5–16] For some sequences, alkylation side reactions can be especially severe.

Dynorphin A is a potent κ-selective endogenous opioid heptadecapeptide that has been isolated from a number of mammalian organs.[17,18] The parent peptide and analogues have been prepared by chemical synthesis[2,11,17,19–25] and are of interest as leads for low-addictive analgesics.[26] For some biological and conformational studies, the C-terminal truncated tridecapeptide analogue has been a

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[1] This paper is dedicated to Professor Ralph Hirschmann on the occasion of his 70th birthday, in recognition of his career with Merck and at the University of Pennsylvania, where he has been responsible for numerous scientific contributions and provided significant leadership on the chemistry and medicinal importance of peptides.

[2] A preliminary account of portions of this work was presented at the 2nd International Symposium on Solid Phase Synthesis, Canterbury, England, Aug 27–31, 1991. Barany, G.; Solé, N. A.; Van Abel, R. J.; Albericio, F.; Selsted, M. E. In *Innovation and Perspectives in Solid Phase Synthesis and Related Technologies: Peptides, Polypeptides and Oligonucleotides 1992*; Epton, R., Ed.; Intercept: Andover, England, 1992; in press.

[3] Abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; CZE, capillary zone electrophoresis; DCM, dichloromethane; DIPCDI, *N,N'*-diisopropylcarbodiimide; DMF, *N,N*-dimethylformamide; EDT, 1,2-ethanedithiol; ESMS, ion electrospray mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; FABMS, fast atom bombardment mass spectrometry; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, reversed-phase high-performance liquid chromatography; MBHA, 4-methylbenzhydrylamine (resin); Nle, norleucine; PAL, tris(alkoxy)-benzylamide linker of ref 6 [5-(4-((9-fluorenylmethyloxycarbonyl)-aminomethyl)-3,5-dimethoxyphenoxy)valeric acid]; Pmc, 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl; PS, copoly(styrene–1%-divinylbenzene) polymeric support; reagent B (ref 2 and this work), TFA–phenol–water–triisopropylsilane (88:5:5:2); reagent K (ref 13), TFA–phenol–water–thioanisole–EDT (82.5:5:5:5:2.5); Reagent R (ref 6), TFA–thioanisole–EDT–anisole (90:5:3:2); SPPS, solid-phase peptide synthesis; *t*Bu, *tert*-butyl; Tmob, 2,4,6-trimethoxybenzyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl); t_R, retention time. Amino acid symbols denote the L-configuration. All solvent ratios and percentages are volume/volume unless stated otherwise.

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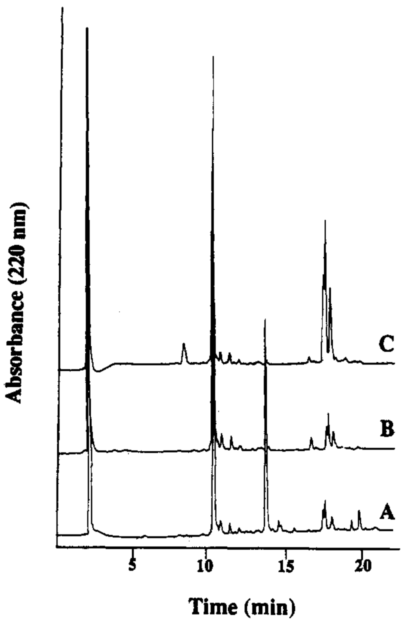

Figure 1. Analytical HPLC of crude [Ala^8]-dynorphin A directly after several "suboptimal" cleavage/workup conditions. See General Procedures for details about experimental and HPLC: (A) crude peptide obtained after cleavage with reagent R (Table I, line 2); (B) crude peptide after cleavage with reagent B, ether precipitation workup (Table I, line 9); and (C) crude peptide after cleavage with modified reagent B that does not have water or phenol (Table I, line 12). The large peaks at the fronts of the chromatograms are solvent; desired peptide elutes at t_R 11.9 min; Tmob-modified dynorphin elutes at t_R 15.8 min, and Pmc-modified dynorphin elutes at t_R 20.5 min.

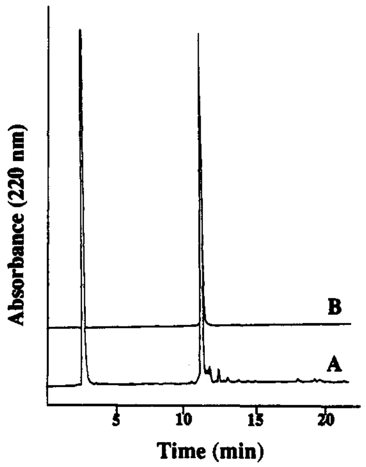

Figure 2. Analytical HPLC of [Ala^8]-dynorphin A reflecting overall conditions of optimized synthesis: (A) crude peptide directly after cleavage with reagent B, extractive workup (Table I, line 10) and (B) purified synthetic peptide after semipreparative HPLC. Other details same as legend to Figure 1.

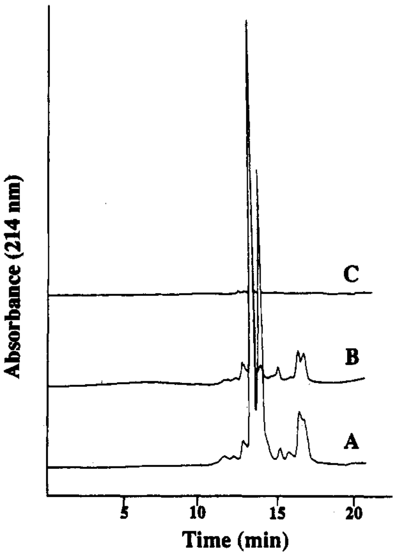

Figure 3. Capillary zone electrophoresis (CZE) of various fractions from this study on [Ala^8]-dynorphin A synthesis. See General Procedures for details about CZE conditions: (A) crude peptide obtained after cleavage with reagent R (Table I, line 2); (B) crude peptide directly after cleavage with reagent B, extractive workup (Table I, line 10); and (C) purified synthetic peptide after semipreparative HPLC. The desired peptide elutes at t_R 7.8 min; Tmob-modified dynorphin elutes at t_R 15.5 min, and Pmc-modified dynorphin elutes at t_R 17.1 min.

relevant target;[17,18,21,22,25] the [Ala^8]-dynorphin A (1-13) analogue (replacing Ile^8 in the native sequence) is particularly potent in an opiate receptor binding assay.[22] During the course of collaborative work to develop efficient synthetic routes in this peptide family for NMR spectroscopic studies (both in aqueous solution and in lipid environments), we recognized that [Ala^8]-dynorphin A (full heptadecapeptide) represented a challenging and sensitive model to probe the usefulness of scavengers, individual or in combination, for the avoidance of alkylation side reactions during acidolysis. The present paper describes the systematic optimization of cleavage and workup conditions and includes the characterization by fast atom bombardment and ion electrospray mass spectrometry (FABMS and ESMS)[6,27,28] of byproducts which form under suboptimal conditions.

Results and Discussion

The linear protected dynorphin A sequence was assembled by Fmoc SPPS with couplings mediated by *N,N'*-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) in *N,N*-dimethylformamide (DMF). To start, N^α-Fmoc, C^α-*tert*-butyl glutamate was attached via the free C^γ-carboxyl group to a "PAL"-resin[3,6] by a DIPCDI/HOBt coupling. The resultant tris(alkoxy)-benzylamide anchoring linkage allows eventual establishment of the C-terminal glutamine residue.[29] Hydrolysis of the completed peptide-resin followed by amino acid analysis revealed ratios in accord with expectation. Furthermore, comparison to a Nle "internal reference" showed that the anchoring step as well as all amino acid incorporation steps were essentially quantitative, with no chain loss throughout the synthesis.

The protected dynorphin A-resin was subjected to an array of pilot-scale cleavage/deprotection reactions (Table I). Cocktails containing principally trifluoroacetic acid as the cleaving agent were examined; these included reagent R,[6] TFA-thioanisole-1,2-ethanedithiol (EDT)-anisole (90:5:3:2), reagent K,[13] TFA-phenol-water-thioanisole-EDT (82.5:5:5:5:2.5), reagent B,[2] TFA-phenol-water-triisopropylsilane (88:5:5:2), and variations. After treatment at 25 °C for the indicated times (1-4 h), the cleaved peptide-resins were filtered, and the crude peptides were obtained from the filtrates by one of two procedures: (i) precipitation with cold diethyl or methyl

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Table I. Optimization of Conditions for Acidolytic Cleavage of [Ala^8]-dynorphin A[a]

				product distribution, %			
line	reagent/scavenger	time, h	cleavage yield, %	peptide (target)	peptide (Tmob)	peptide (Pmc)	others
1	TFA–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	1*	63	53	37	5	4
2	TFA–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	1	60	54	44	<1	–
3	TFA–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	2	69	82	17	<1	<1
4	TFA–thioanisole–EDT–anisole (90:5:3:2) (reagent R)	4	81	86	13	<1	<1
5	TFA–phenol–water–thioanisole–EDT (82.5:5:5:5:2.5) (reagent K)	1*	69	60	38	<1	<1
6	TFA–phenol–water–thioanisole–EDT (82.5:5:5:5:2.5) (reagent K)	1	65	69	20	1	10
7	TFA–phenol–water–thioanisole–EDT (82.5:5:5:5:2.5) (reagent K)	2	73	87	10	<1	<1
8	TFA–phenol–water–thioanisole–EDT (82.5:5:5:5:2.5) (reagent K)	4	83	90	7	<1	2
9	TFA–phenol–water–triisopropylsilane (88:5:5:2) (reagent B)	1*	97	78	–	6	5
10	TFA–phenol–water–triisopropylsilane (88:5:5:2) (reagent B)	1	96	93	–	1	6
11	TFA–phenol–triisopropylsilane (93:5:2) (reagent B without water)	1	96	90	–	4	6
12	TFA–triisopropylsilane (98:2) (reagent B without water, phenol)	1	95	52	–	20	23

[a] All cleavage reactions were carried out at 25 °C, using the freshly prepared cleavage cocktail listed in column 2 for the time indicated in column 3. An asterisk (*) next to the time means an ether precipitation was carried out; in all others cases, an extractive workup was used (details in text). Cleavage yields in column 4 are calculated from the ratio of amino acids in the cleaved resin, with repeat to Nle "internal reference" amino acid incorporated onto resin before the PAL handle. The crude product mixtures from the cleavages were subjected directly to HPLC analysis (e.g., Figure 1), and the product distributions reported are *uncorrected* for relative absorbances of peptides. Identities of the target and Tmob-alkylated peptide were confirmed by semipreparative isolation. The Pmc-peptides were in a region of the HPLC chromatogram that included additional peaks which are listed under "other"; the "other" tabulation also includes shoulders and satellites to the already identified peaks.

tert-butyl ether or (ii) dilution with aqueous acetic acid, followed by ether extraction and lyophilization of the aqueous phase. These crude peptide products were then evaluated by analytical high-performance liquid chromatography (HPLC; e.g., Figures 1 and 2) and capillary zone electrophoresis (CZE; e.g., Figure 3), as well as direct FABMS or ESMS. Under some conditions, substantial levels of Tmob-modified dynorphin [FABMS found monoisotopic MH$^+$ of 2285.3 vs calculated 2285.2; additional peaks in isotope cluster due to one or more ^{13}C were observed for this and other peptides in a ratio which agreed with statistical prediction; ESMS found average mass of 2285.6 ± 0.6 vs calculated 2285.7] and Pmc-modified dynorphin [FABMS monoisotopic MH$^+$ of 2372.0 found vs 2371.2 calculated; ESMS average mass of 2371.4 ± 0.4 found vs calculated 2371.8] were present, together with the desired dynorphin [FABMS monoisotopic MH$^+$ of 2105.8 found vs 2105.2 calculated; ESMS average mass of 2105.6 ± 0.6 found vs calculated 2105.5].[30]

Cleavage of the protected dynorphin A-resin with reagents R and K proceeded in 60–80% yield, as judged by hydrolysis and amino acid analysis of the cleaved resin, with respect to the Nle "internal reference" (Table I, lines 1–8). Retention of peptide on the support is most likely due to a precedented back-addition process involving alkylation of the Trp residue with the resin-bound tris(alkoxy)benzylcarbonium ion.[6] As for the peptide mixture released into solution, the major byproduct was dynorphin with its Trp residue alkylated by Tmob from the single Asn residue two over in the sequence. The level of the Tmob modification was substantial (up to 40%) after 1 h of cleavage, but *abated* considerably with longer cleavage. Such initial alkylation, followed by a slower reversal, has been described previously.[13] Finally, the scavengers present in reagents R and K were quite efficient in preventing modification of Trp by any of the three Pmc groups in the protected dynorphin sequence.

The overall *best* results were obtained upon cleavage with reagent B, particularly when extraction rather than precipitation was used for workup (Table I, line 10 vs line 9; see also Figure 1, panel C vs Figure 2, panel A and Figure 3, panel B). Cleavage yields were consistently in the 95% range, and the soluble Tmob alkylation side reaction noted earlier was entirely avoided. Thus, silanes are very effective as traps for tris(alkoxy)benzyl carbocations.[2,8,31] With the optimal reagent B, Pmc modification of Trp was negligible. However, when water and particularly phenol were omitted from the cocktail, Pmc-modified dynorphin formed at high levels (5–20% or more; see Table I, lines 11 and 12). Replacement of triisopropylsilane by either triethylsilane or triisobutylsilane gave results which were qualitatively similar (data not shown).[32]

On the basis of these results, reagent B cleavage and extractive workup was repeated on a larger scale, and semipreparative HPLC gave [Ala^8]-dynorphin A in excellent purity (Figure 2, panel B; Figure 3, panel C) and 58% overall isolated yield based on the C-terminal amino acid anchored on the resin. The purified peptide coeluted with commercially available material upon HPLC and CZE, and showed satisfactory FABMS and ESMS spectra completely free of extraneous peaks noted in crude synthetic dynorphin.

Conclusions

The experiments reported in this paper have led to an efficient preparation of [Ala^8]-dynorphin A in high overall yield and purity by Fmoc chemistry. An important feature

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(30) The Tmob-modified peptide was isolated in pure form (light purple!) by semipreparative HPLC, and gave the expected FABMS and ESMS spectra. In some cases, the FABMS of unfractionated peptide mixtures also included peaks corresponding to dynorphin with both Tmob and Pmc [monoisotopic MH$^+$ of 2552.3] or with two Pmc [monoisotopic MH$^+$ of 2637.3]. These low-level byproducts, not associated with any discrete HPLC peak, presumably represent *incomplete* deprotection *along with* alkylation at Trp. Also, an unidentified FABMS peak at m/z 2203.2 (97.4 above desired dynorphin) was sometimes seen in those crude peptide products that had been worked up by text procedure i, though not by procedure ii. We suspect that this peak may be a FABMS mass spectrometric artifact due to the presence of residual TFA in ether-precipitated peptides. The corresponding problem was never seen upon ESMS.

(31) Our current recommendation for reagent B uses 2% triisopropylsilane (v/v), i.e., 0.1 M. Under the cleavage conditions reported, the silane is in 50-fold excess over the protected peptide-resin or 5-fold excess over protecting groups and handle which can give rise to carbonium ions. Lower amounts of silane can be effective, so long as some excess is maintained over the groups which are to be scavenged.

(32) In ref 12, Pearson et al. counsel against the use of triethylsilane, which could reduce the indole nucleus of Trp. However, this reagent is included in the cocktail of ref 16. On the dynorphin model of the present work, the major byproducts were the same regardless of which silane was used (presence or absence of water and/or phenol); extra low-level peaks were found with triethylsilane.

of our synthetic strategy involves side-chain anchoring to a PAL-resin, thereby preventing problems which would otherwise be anticipated to occur with C-terminal glutamine.[29] Our studies on the optimization of the scavenger cocktails and reaction times for acidolytic deprotection and cleavage, and our observations regarding subsequent workup conditions, reinforce the conventional wisdom[5,33] that the listed experimental parameters require careful attention in order to maximize the amounts of desired peptide formed upon SPPS. Reagents R, K, and B each have meritorious features; reagent B can be recommended in part due to the absence of malodorous components in its formulation and use. Our results should be generalizable to other sensitive peptides, from within as well as from outside of the dynorphin family.

Experimental Section

General Procedures. Some of the materials and general synthetic and analytical procedures have been described in earlier publications from our laboratory.[2,6,8,29,34] Analytical HPLC was performed using a Vydac analytical C-18 reversed-phase column (218TP54, 5 μm, 300 Å; 0.46 × 25 cm) on a Beckman system configured with two Model 112 pumps and a Model 165 variable-wavelength detector controlled from an IBM computer with Beckman System Gold software. Peptide samples were chromatographed at 1.2 mL/min using 0.012 N aqueous HCl–CH_3CN (9:1 to 6:4 over 20 min), detection at 220 nm. Semipreparative HPLC was performed using a Vydac semipreparative C-18 reversed-phase column (218TP1010; 10 μm, 300 Å; 1.0 × 25 cm) on a Waters Deltaprep system using manual injection (1 mL, ~5 mg of peptide per run) and elution at 6 mL/min using 0.012 N aqueous HCl–CH_3CN (9:1–6:4 over 60 min), detection at 280 nm. Capillary zone electrophoresis (CZE) was performed on a Beckman P/ACE System 2100 in capillary cartridges (fused silica 75-μm i.d. × 50-cm length; 100 × 200 aperture), as follows: prewash for 1 min with 0.1 M sodium phosphate (pH 2.0); equilibration of capillary for 2 min with running buffer, 50 mM sodium phosphate (pH 2.0)–CH_3CN (9:1); 10-s pressure injection; and separation for 20 min at 15 kV and 90 mamps; detection at 214 nm. Positive-ion fast atom bombardment mass spectrometry (FABMS) was carried out on a VG 7070E-HF instrument, scans from 500 to 3000/300 s/decade, operated at a resolution of 3000. Matrices of glycerol–water, thioglycerol–water, or *m*-nitrobenzyl alcohol–water were used with the various crude and purified synthetic peptide products which were characterized by FABMS in this work. Ion electrospray mass spectra (ESMS) were determined on a Sciex API III mass spectrometer using an Ionspray interface. Molecular masses were calculated with the Sciex Hypermass program from m/z values of ions containing 2–4 charges. Samples were injected from a syringe pump at 10 μL/min; for ESMS, it was possible to use directly cleavage mixtures, effluents from HPLC preparative purifications, or lyophilized peptides which were redissolved in 0.1% aqueous formic acid–CH_3CN (1:1).

Protected Fmoc-amino acid derivatives were from Bachem Bioscience (Philadelphia, PA) or Advanced Chemtech (Louisville, KY). The handle-resin Fmoc-PAL-Nle-MBHA-PS was from Millipore (Bedford, MA; formerly MilliGen/Biosearch in Novato, CA). *N,N*-Dimethylformamide (DMF) was "sequencing grade" from Fisher (Pittsburgh, PA), and was flushed with N_2 for at least 1 day and maintained over Amberlyst-15 (H-form) ion-exchange resin prior to its use for peptide synthesis (confirmed negative to FDNB test, carried out according to ref 35). Piperidine, trifluoroacetic acid (TFA), and 1-hydroxybenzotriazole (HOBt) were also sequencing grade from Fisher, and *N,N'*-diisopropylcarbodiimide (DIPCDI) as well as all scavengers were obtained from Aldrich (Milwaukee, WI). Peptide-resins were hydrolyzed in 12 N HCl–propionic acid (1:1 v/v) + 2 drops liquified phenol at 130 °C for overnight or 160 °C for 2 h.

Assembly of Protected [Ala^8]-dynorphin A-Resin: H-Tyr(*t*Bu)-Gly-Gly-Phe-Leu-Arg(Pmc)-Arg(Pmc)-Ala-Arg(Pmc)-Pro-Lys(Boc)-Leu-Lys(Boc)-Trp-Asp(O*t*Bu)-Asn(Tmob)-Glu(PAL-resin)-O*t*Bu. The synthesis was carried out manually, starting with an Fmoc-PAL-Nle-MBHA resin (0.2 g, 0.3 mmol amino sites/g, 0.06 mmol) and using appropriate N^α-Fmoc-amino acid derivatives (0.24 mmol each, 4.0 equiv). The overall synthetic design and protection strategy is discussed in the text, and experimental procedures were similar to those reported earlier from our laboratories. Fmoc removal was with piperidine–DMF (1:4, 2 × 8 min), followed by washing with DMF–DCM (1:1, 4 × 2 min) and DMF (4 × 2 min) (volume of 1.5 mL for deprotection and all washing steps). Couplings were achieved by combining, under N_2, solid N^α-Fmoc-amino acids and HOBt (4 equiv each) with a 0.5 M solution of DIPCDI in DMF (0.5 mL), waiting 10 min for preactivation at 25 °C, and then adding this solution to the peptide resin. Single or double coupling were carried out for 1–2 h,[36] at which point manual ninhydrin tests[37] on resin aliquots were always negative. Upon completion of chain assembly, the protected peptide-resin (0.3 g; note that samples were removed for ninhydrin tests and amino acid analysis) was dried for 24 h in vacuo (1 mm). Hydrolysis and amino acid analysis of the peptide-resin showed the expected ratios: Asx 2.11; Glx 1.03; Pro 1.02; Gly 2.00; Ala 1.01; Leu 2.07; Tyr 0.90; Phe 0.83; Lys 1.92; Arg 3.01; Nle 1.01; and a final substitution level (0.15 mmol chains/g peptide-resin) in accord with expectation for quantitative chain assembly and no loss of chains from the support.

Pilot Cleavage Studies (Table I). Cleavage cocktails were prepared freshly before use and degassed with N_2. Portions of protected peptide-resin (20–30 mg) were placed into manual reaction vessels (stationary plastic syringes fitted with filter frits), preswollen, and washed with DCM (5 × 3 mL), and the cleavage cocktails (3 mL) were added. After the indicated time for overhead stirring at 25 °C, the cleaved peptide-resins were filtered and washed with a small amount of further cocktail (0.5 mL). The combined filtrates were cooled to 4 °C and treated further in one of the following ways: (i) ice-cold diethyl ether or methyl *tert*-butyl ether (10 mL) was added with thorough stirring. After ~10 min, the precipitated peptide product was collected by low-speed centrifugation, washed with cold ether (4 × 10 mL), and dissolved in 20% aqueous HOAc (8 mL), or (ii) dilution with 20% aqueous HOAc (8 mL), followed by extraction with ice-cold diethyl ether or methyl *tert*-butyl ether (5 × 5 mL). The aqueous acetic acid solutions of crude peptide were injected directly for HPLC and CZE analysis (Figures 1–3) or for ESMS; for FABMS or further procedures, these solutions were lyophilized.

[Ala^8]-dynorphin A: H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ala-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH. The conditions of Table I, line 10 (see also Figure 2, panel A) were applied with 40 mg of protected peptide-resin (6 μmol) to provide crude peptide (12 mg) after lyophilization. A portion of this material (nominally 7 mg; 5.25 mg based on amino acid analysis) was applied to semipreparative HPLC (see General Procedures). Fractions with the correct peptide were pooled and lyophilized to provide a white powder (3.2 mg, 1.5 μmol, 61% isolated yield for preparative chromatography) which was pure by HPLC (Figure 2, panel B) and CZE (Figure 3, panel C). Hydrolysis and amino acid analysis of the peptide showed the expected ratios: Asx 2.02; Glx 1.06; Pro 1.08; Gly 2.05; Ala 0.99; Leu 1.97; Tyr 0.90; Phe 0.83; Lys 1.94; Arg 3.01. FABMS showed the expected quasimolecular ion cluster, largest peak at m/z 2106.8 (includes one ^{13}C), and none of the ions due to synthetic byproducts as discussed in text. There was also an intense cluster starting at m/z 1052.3, due to fragmentation at Pro. ESMS showed an average mass of 2105.6 ± 0.6. A similar procedure carried out on the cleaved material from the conditions of Table I, line 1, allowed isolation of Tmob-alkylated dynorphin, which was pure by HPLC and CZE (data not

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[33] A particularly interesting cautionary example regarding workup of Trp-containing peptides is reported by: Ponsati, B.; Giralt, E.; Andreu, D. In *Peptides: Chemistry, Structure and Biology*; Rivier, J. E., Marshall, G. R., Eds.; Escom: Leiden, The Netherlands, 1990; pp 960–962.

[34] Ferrer, M.; Woodward, C.; Barany, G. *Int. J. Peptide Protein Res.* **1992**, *40*, in press.

[35] Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.; Rockford, IL, 1984; pp 69–70.

[36] After 1 h coupling, ninhydrin tests (ref 37) were slightly positive for Lys^{13}, Arg^9 through Arg^6, as well as Gly^3 and Tyr^1. For all of these residues, fresh activated amino acid was added for a second 1 h coupling.

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shown), and characterized further by FABMS (clusters starting at 2285.3 and 1052.3) and ESMS (average mass 2285.6 ± 0.6). In the same way, Pmc-alkylated dynorphin was isolated (~90% pure by HPLC), and characterized by ESMS (average mass 2371.4 ± 0.4).

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Lewis Acid Promoted Stereoselective Carbon–Carbon Bond Formation of 3-Formyl-Δ^2-isoxazolines

Akio Kamimura,* Kosei Yoshihara, Shinji Marumo, Akinori Yamamoto, Takeshi Nishiguchi, Akikazu Kakehi,† and Kenzi Hori

Department of Chemistry, Faculty of Liberal Arts, Yamaguchi University, Yamaguchi 753, Japan, and Department of Chemistry and Material Engineering, Faculty of Engineering, Shinshu University, Nagano 380, Japan

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4,5-Disubstituted 3-formyl-Δ^2-isoxazolines undergo the aldol, allylation, and carbonyl ene reactions in the presence of appropriate Lewis acid to give the adducts with an effective 1,3-asymmetric induction. The stereoselectivity of the reaction mainly depends on the nature of the Lewis acid and the relative configuration of the ring. It is remarkable that both diastereomers can be readily prepared stereoselectively. For example, $TiCl_4$ promotes the 1,3-syn-selective aldol reaction over 93/7 of selectivity, while the 1,3-anti adducts are prepared by the reaction catalyzed by $BF_3{\cdot}OEt_2$. This difference in stereoselectivity is to be attributed to the preferable conformation of isoxazoline–Lewis acid complex intermediates, which depends on the nature of Lewis acid. Without the 4-substituent of isoxazolines the selectivity is not observed. The 5-substituent is too far from the formyl carbon to influence the face differentiation of the formyl group. Subsequent treatment of the adducts with $LiAlH_4$ affords 2-amino 1,4-diol derivatives. The protective group of the hydroxyl group on the C(3) side chain is crucial for the stereoselectivity of the reduction. An almost complete diastereoselectivity of the relative configuration at four contiguous stereogenic centers is readily achieved by the reduction of the adducts protected by *O*-methoxymethyl (O-MOM). Consequently, the present strategy provides a facile method for the preparation of the compounds containing a sequence of several stereocenters.

Introduction

Δ^2-Isoxazolines (4,5-dihydroisoxazoles) have been found to be useful intermediates in organic synthesis as a result of the development of their chemistry for the past decade.[1] We can summarize their synthetic utilities as follows: easy preparation, diversity of their conversion, and characteristic ring structure. Firstly, they are readily prepared via an inter- or an intramolecular 1,3-dipolar cycloaddition of nitrile oxides.[1a,h] It is also an important feature that the relative configuration of the ring substituents can be readily controlled by choosing the geometries of starting olefins, since the 1,3-dipolar cycloaddition of nitrile oxides generally takes place in a highly stereoselective manner. Alternatively, introduction of a 4-substituent of the ring is readily achieved via Jäger alkylation in a highly C(4)/C(5) trans selective way.[2] Secondly, they can be converted into various important synthetic units such as β-hydroxy ketones[3] or γ-amino alcohols[4] without the loss of stereochemistry of the ring substituents.[1b–d,f] This feature characterizes the isoxazoline synthesis as an alternative stereoselective aldol method.[1f] Additionally, Jäger et al. extensively studied the stereoselective conversion of the ring into γ-amino alcohols, and with this strategy they accomplished total synthesis of amino sugar derivatives.[5,6] Finally, the isoxazoline ring has a characteristic structure in a relatively rigid five-membered ring, containing two heteroatoms which serve as Lewis bases. The last feature is supposed to be useful in developing methods for controlling the stereochemistry of the carbon adjacent to the ring. So far there have been several reports concerning this issue,[7] whereas very scarcely has there been reported

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† Shinshu University.

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