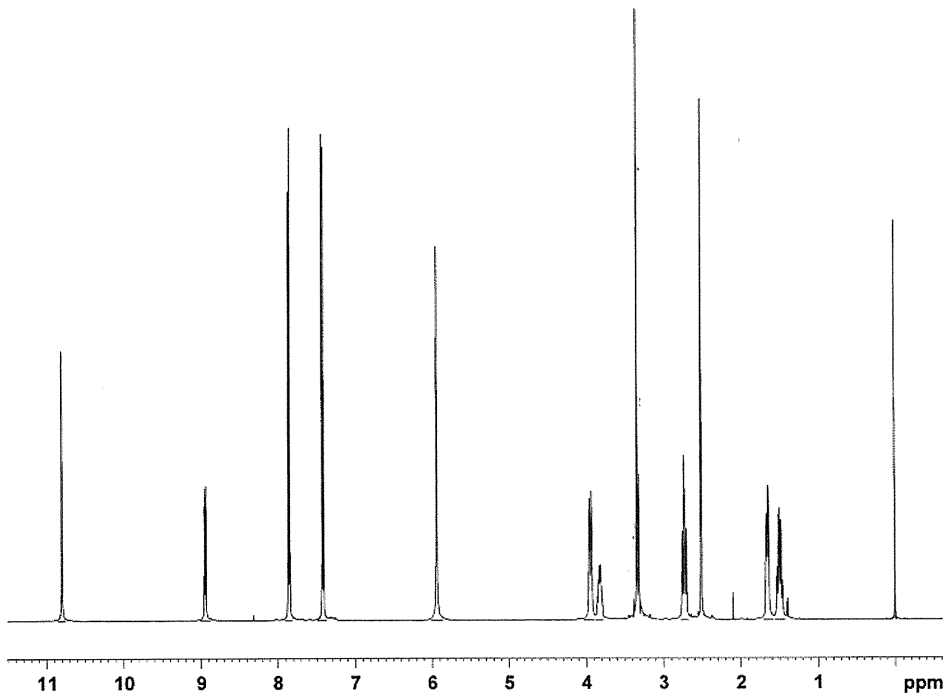


Compound 6

<sup>1</sup>H NMR

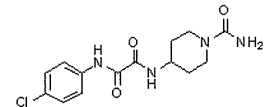


IBB-nmr Analysis

```

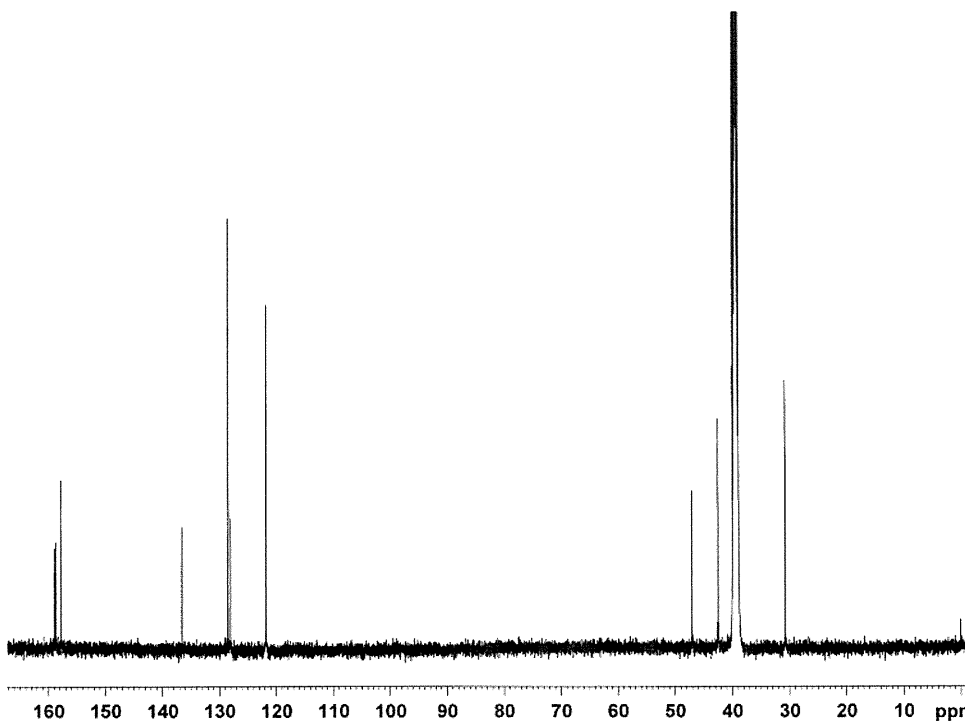
NAME          HAR2-44
EXPNO         2
PROCNO        1
Date_         20110514
Time          14.30
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zg30
TD            65536
SOLVENT       DMSO
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1720407 sec
RG            22.6
DW            48.400 usec
DE            6.00 usec
TE            298.0 K
D1            1.0000000 sec
TD0           1

----- CHANNEL f1 -----
NUC1          1H
P1            10.00 usec
PL1           -4.60 dB
PL1W          7.24435949 W
SF01          500.1330885 MHz
SI            32768
SF            500.1300000 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            0.50
    
```



Compound 6

<sup>13</sup>C NMR



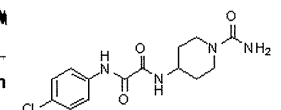
IBB-nmr Analysis

```

NAME          HAR2-44
EXPNO         4
PROCNO        1
Date_         20110514
Time          14.43
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zgpg30
TD            65536
SOLVENT       DMSO
NS            64
DS            4
SWH           30030.029 Hz
FIDRES        0.458222 Hz
AQ            1.0912410 sec
RG            90.5
DW            16.650 usec
DE            20.00 usec
TE            298.0 K
D1            2.0000000 sec
D11           0.0300000 sec
TD0           1

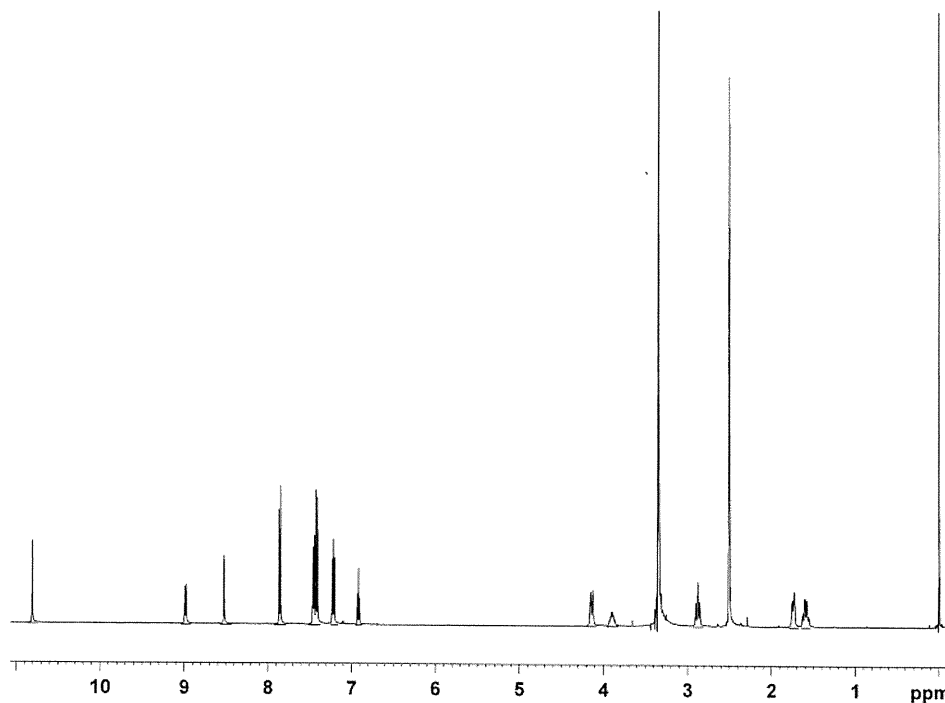
----- CHANNEL f1 -----
NUC1          13C
P1            10.00 usec
PL1           -4.80 dB
PL1W          13.65439701 W
SF01          125.7703643 MHz

===== CHANNEL f2 =====
CPDPRG2       waltz16
NUC2          1H
PCPD2         80.00 usec
PL2           -4.80 dB
PL12          12.80 dB
PL13          16.00 dB
PL2W          7.58577585 W
PL12W         0.13182567 W
PL13W         0.06309573 W
SF02          500.1320005 MHz
SI            32768
SF            125.7578519 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            0.30
    
```



Compound 7

<sup>1</sup>H NMR



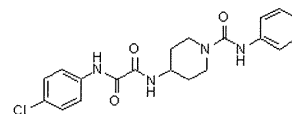
*IBB-nmr Analysis*

```

NAME          HAR2-34
EXPNO         4
PROCNO        1
Date_         20110609
Time_         15.32
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zg30
TD            65536
SOLVENT       DMSO
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1720407 sec
RG            22.6
DW            48.400 usec
DE            6.00 usec
TE            298.0 K
D1            1.0000000 sec
D11           1
TDO           1
    
```

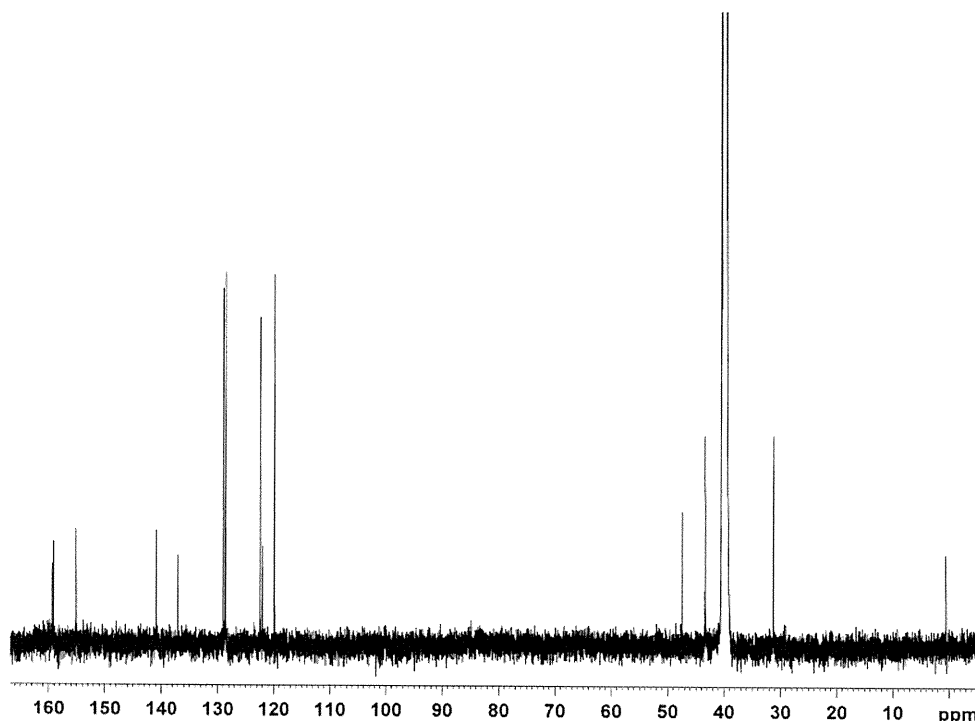
```

===== CHANNEL f1 =====
NUC1          1H
P1            10.00 usec
PL1           -4.60 dB
PL1W          7.24435949 W
SFO1          500.1330885 MHz
SI            32768
SF            500.1300048 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            0.50
    
```



Compound 7

<sup>13</sup>C NMR



*IBB-nmr Analysis*

```

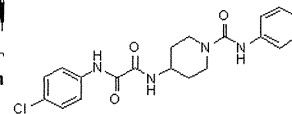
NAME          HAR2-34
EXPNO         6
PROCNO        1
Date_         20110615
Time_         10.17
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zgpg30
TD            65536
SOLVENT       DMSO
NS            64
DS            4
SWH           30030.029 Hz
FIDRES        0.458222 Hz
AQ            1.0912410 sec
RG            90.5
DW            16.650 usec
DE            20.00 usec
TE            298.0 K
D1            2.0000000 sec
D11           0.03000000 sec
TDO           1
    
```

```

===== CHANNEL f1 =====
NUC1          13C
P1            10.00 usec
PL1           -4.80 dB
PL1W          13.65439701 W
SFO1          125.7703643 MHz
    
```

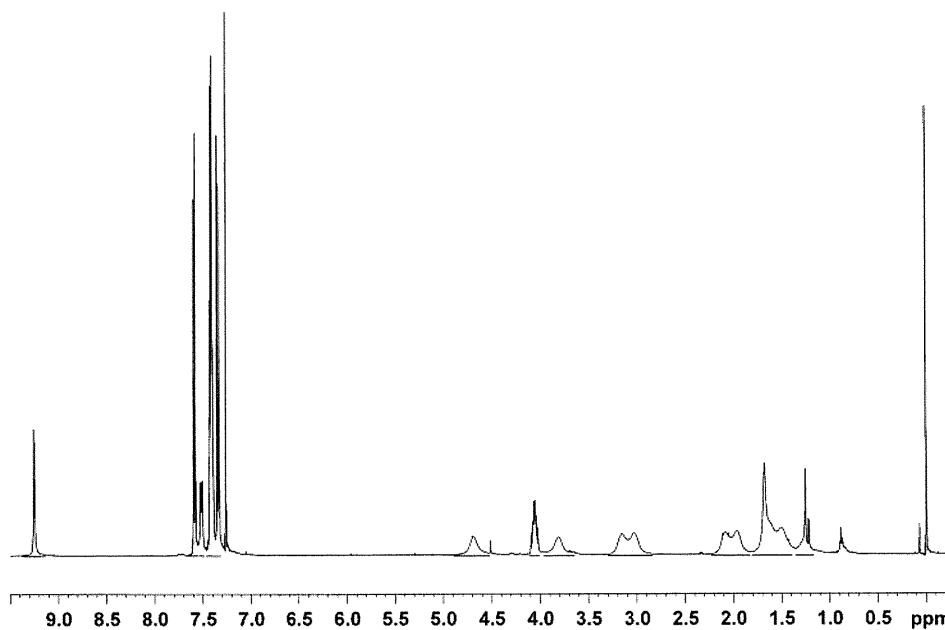
```

===== CHANNEL f2 =====
PCPDPRG2     waltz16
NUC2          1H
PCPD2        80.00 usec
PL2           -4.80 dB
PL12          12.80 dB
PL13          16.00 dB
PL2W          7.58577585 W
PL12W         0.13182567 W
PL13W         0.06309573 W
SFO2          500.1320005 MHz
SI            32768
SF            125.7577932 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            0.30
    
```



Compound 8

<sup>1</sup>H NMR



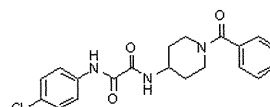
IBB-nmr Analysis

```

NAME          HAR2-14
EXPNO         6
PROCNO        1
Date_         20110621
Time          17.02
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zg30
TD            65536
SOLVENT       CDCl3
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1720407 sec
RG            14.3
DM            48.400 usec
DE            6.00 usec
TE            298.0 K
D1            1.0000000 sec
TD0           1
    
```

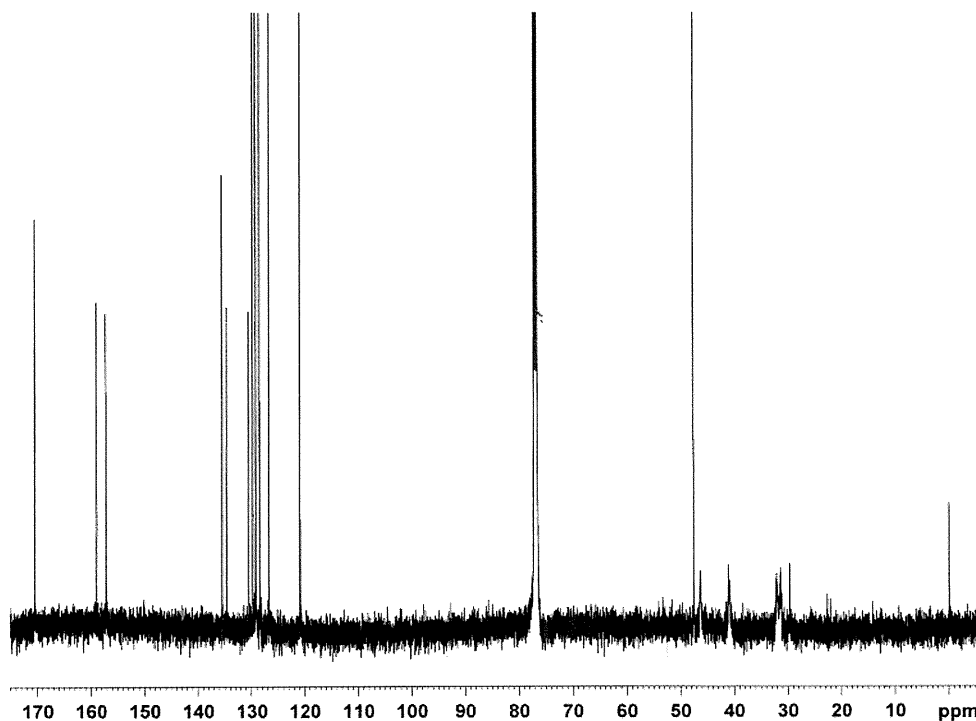
```

===== CHANNEL f1 =====
NUC1          1H
P1            10.00 usec
PL1           -4.60 dB
PL1W          7.24435949 W
SFO1          500.1330885 MHz
SI            32768
SF            500.1300111 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            0.50
    
```



Compound 8

<sup>13</sup>C NMR



IBB-nmr Analysis

```

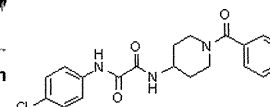
NAME          HAR2-14
EXPNO         7
PROCNO        1
Date_         20110621
Time          17.09
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zgpg30
TD            65536
SOLVENT       CDCl3
NS            64
DS            4
SWH           30030.029 Hz
FIDRES        0.458222 Hz
AQ            1.0912410 sec
RG            90.5
DM            16.650 usec
DE            20.00 usec
TE            298.0 K
D1            2.0000000 sec
D11           0.0300000 sec
TD0           1
    
```

```

===== CHANNEL f1 =====
NUC1          13C
P1            10.00 usec
PL1           -4.80 dB
PL1W          13.65439701 W
SFO1          125.7703643 MHz
    
```

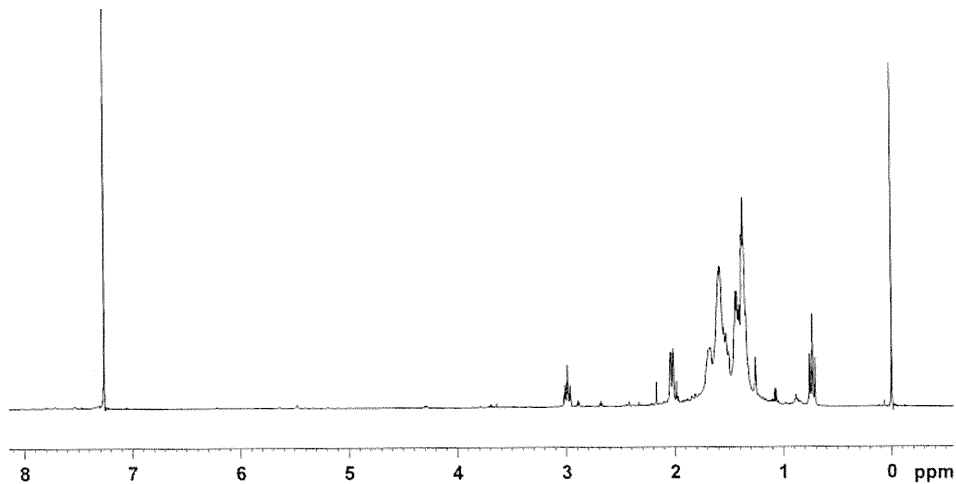
```

===== CHANNEL f2 =====
CPDPRG2       waltz16
NUC2          1H
PCPD2         80.00 usec
PL2           -4.80 dB
PL12          12.80 dB
PL13          16.00 dB
PL2W          7.58577585 W
PL12W         0.13182567 W
PL13W         0.06309573 W
SFO2          500.1320005 MHz
SI            32768
SF            125.7577932 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            0.30
    
```



Compound 10

<sup>1</sup>H NMR



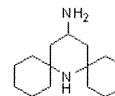
IBB-nmr Analysis

```

NAME          HAR4-22
EXPNO         20
PROCNO        1
Date_         20110801
Time_        11.03
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zg30
TD            65536
SOLVENT       CDCl3
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1720407 sec
RG            14.3
DW            48.406 usec
DE            6.00 usec
TE            298.0 K
D1            1.0000000 sec
TD0           1
    
```

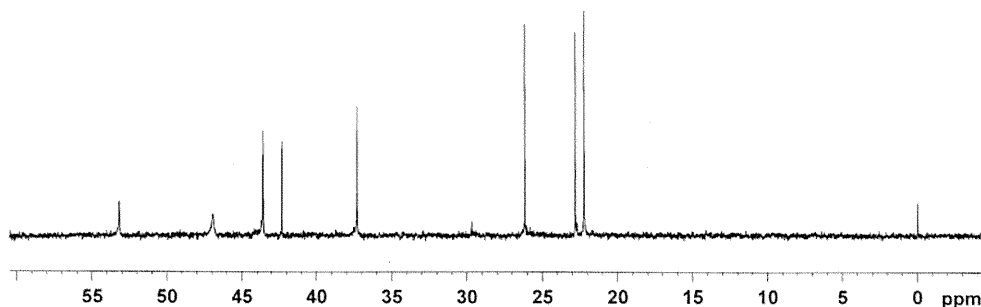
```

===== CHANNEL f1 =====
NUC1          1H
P1            10.00 usec
PL1           -4.60 dB
PL1W          7.24435949 W
SFO1          500.1330885 MHz
SI            32768
SF            500.1300106 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            0.50
    
```



Compound 10

<sup>13</sup>C NMR



IBB-nmr Analysis

```

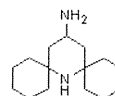
NAME          HAR4-22
EXPNO         21
PROCNO        1
Date_         20110801
Time_        11.09
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zgpg30
TD            65536
SOLVENT       cdcl3
NS            64
DS            4
SWH           30030.029 Hz
FIDRES        0.458222 Hz
AQ            1.0912410 sec
RG            128
DW            16.650 usec
DE            20.00 usec
TE            298.0 K
D1            2.0000000 sec
D11           0.0300000 sec
TD0           1
    
```

```

===== CHANNEL f1 =====
NUC1          13C
P1            10.00 usec
PL1           -4.80 dB
PL1W          13.65439701 W
SFO1          125.7703643 MHz
    
```

```

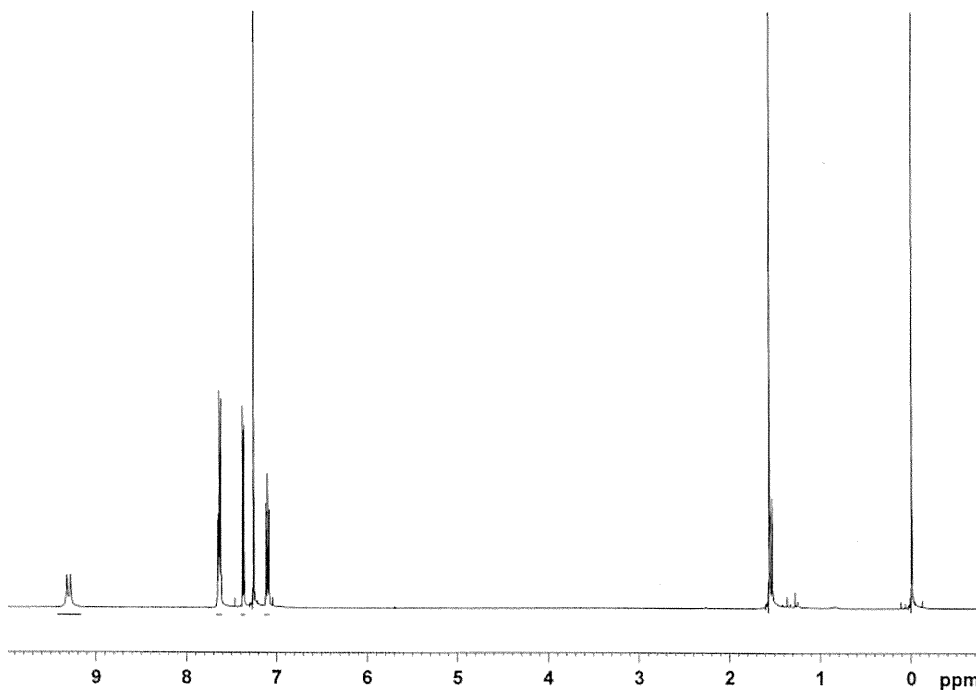
===== CHANNEL f2 =====
CPDPRG2       waltz16
NUC2          1H
PCPD2         80.00 usec
PL2           -4.80 dB
PL12          12.80 dB
PL13          16.00 dB
PL2W          7.58577585 W
PL12W         0.13182567 W
PL13W         0.06309573 W
SFO2          500.1320005 MHz
SI            32768
SF            125.7577932 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            0.30
    
```





Compound 14

<sup>1</sup>H NMR



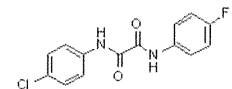
*IBB-nmr Analysis*

```

NAME          HARI-14
EXPNO         1
PROCNO        1
Date_         20110711
Time          19.39
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zg30
TD            65536
SOLVENT       CDCl3
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1720407 sec
RG            25.4
DW            40.400 usec
DE            6.00 usec
TE            298.0 K
D1            1.0000000 sec
D10           1
    
```

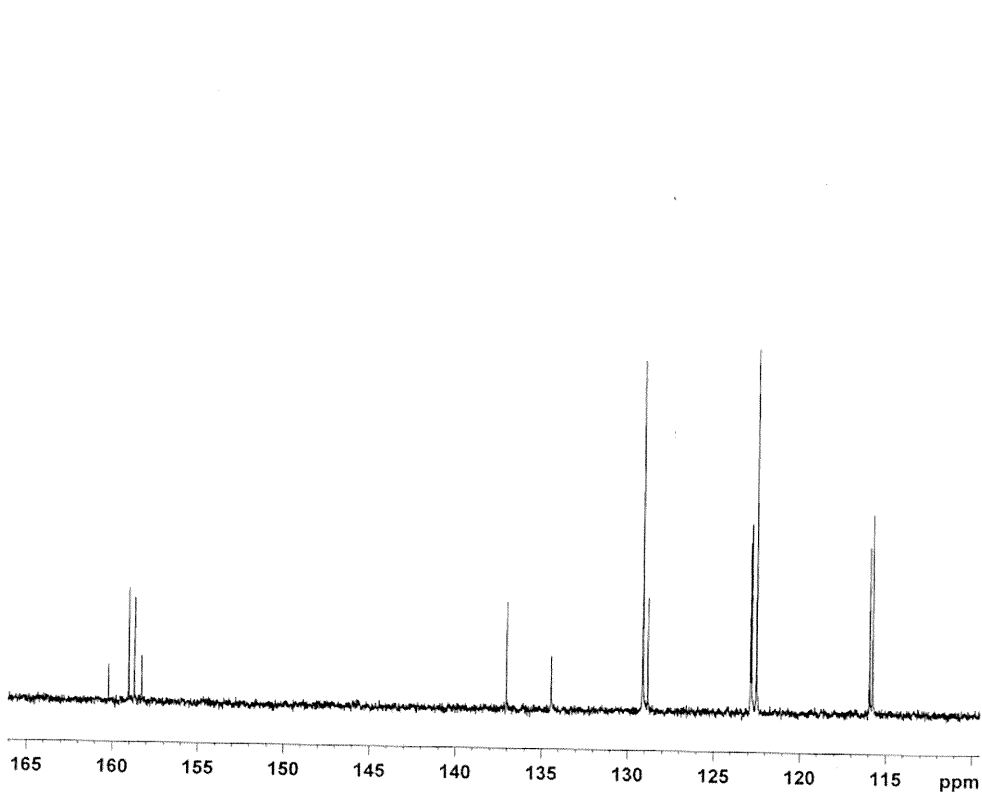
```

===== CHANNEL f1 =====
NUC1          1H
P1            10.00 usec
PL1           -4.60 dB
PL1W          7.24435949 W
SFO1          500.1330885 MHz
SI            32768
SF            500.1300124 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            0.50
    
```



Compound 14

<sup>13</sup>C NMR



*IBB-nmr Analysis*

```

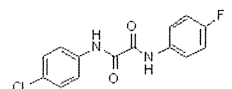
NAME          HARI-14
EXPNO         2
PROCNO        1
Date_         20110712
Time          19.55
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zgpg30
TD            65536
SOLVENT       DMSO
NS            64
DS            4
SWH           30030.029 Hz
FIDRES        0.458222 Hz
AQ            1.0912410 sec
RG            90.5
DW            16.650 usec
DE            20.00 usec
TE            298.0 K
D1            2.0000000 sec
D11           0.0300000 sec
D10           1
    
```

```

===== CHANNEL f1 =====
NUC1          13C
P1            10.00 usec
PL1           -4.80 dB
PL1W          13.65439701 W
SFO1          125.7703643 MHz
    
```

```

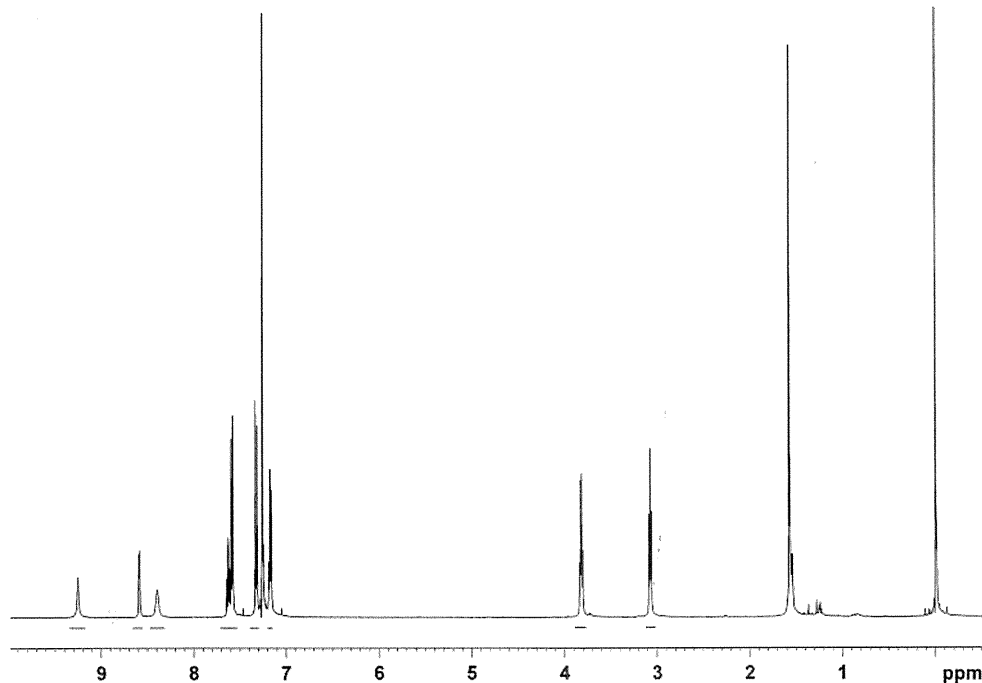
===== CHANNEL f2 =====
CPDPRG2       waltz16
NUC2          1H
PCPD2         80.00 usec
PL2           -4.80 dB
PL12          12.80 dB
PL13          16.00 dB
PL2W          7.58577585 W
PL12W         0.13182567 W
PL13W         0.06309573 W
SFO2          500.1320005 MHz
SI            32768
SF            125.7577932 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            0.30
    
```



Compound 17

<sup>1</sup>H NMR

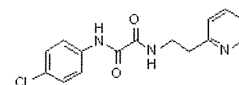
IBB-nmr Analysis



```

NAME          HARI-60
EXPNO         1
PROCNO        1
Date_         20110711
Time_         19.50
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zg30
TD            65536
SOLVENT       CDCl3
NS            16
DS            2
SWH           10330.578 Hz
FIDRES        0.157632 Hz
AQ            3.1720407 sec
RG            28.5
DW            48.400 usec
DE            6.00 usec
TE            298.0 K
D1            1.0000000 sec
D11           1
TDO           1

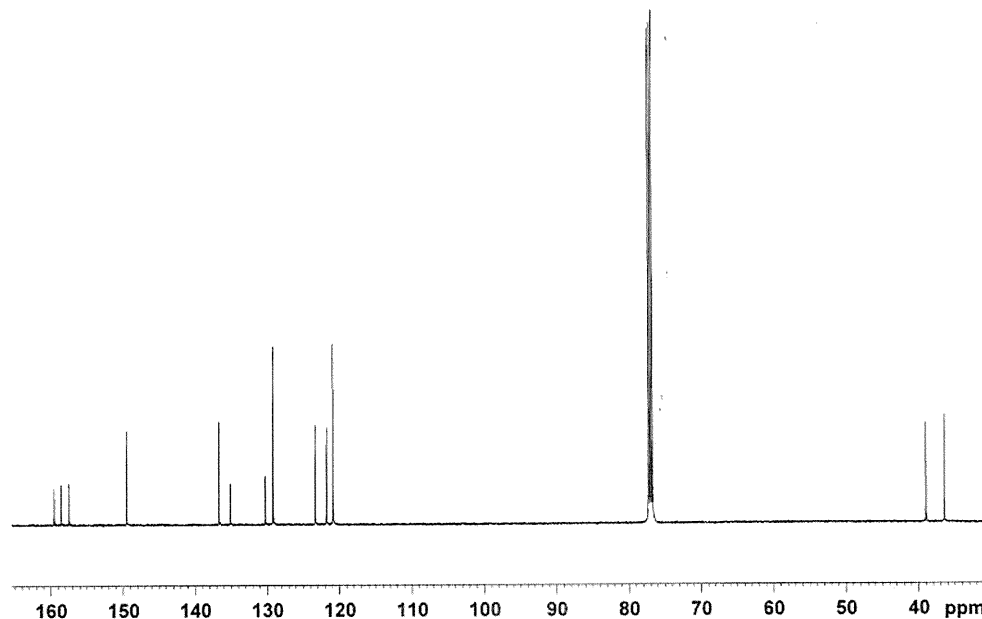
===== CHANNEL f1 =====
NUC1          1H
P1            10.00 usec
PL1           -4.60 dB
PL1W          7.24435949 W
SFO1          500.1330885 MHz
SI            32768
SF            500.1300122 MHz
WDW           EM
SSB           0
LB            0.30 Hz
GB            0
PC            0.50
    
```



Compound 17

<sup>13</sup>C NMR

IBB-nmr Analysis

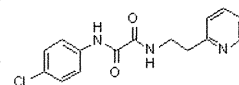


```

NAME          HARI-60
EXPNO         2
PROCNO        1
Date_         20110711
Time_         20.23
INSTRUM       av500
PROBHD        5 mm CPDCH 13C
PULPROG       zgpg30
TD            65536
SOLVENT       CDCl3
NS            64
DS            4
SWH           30030.029 Hz
FIDRES        0.458222 Hz
AQ            1.0912410 sec
RG            90.5
DW            16.650 usec
DE            20.00 usec
TE            298.0 K
D1            2.0000000 sec
D11           0.0300000 sec
TDO           1

===== CHANNEL f1 =====
NUC1          13C
P1            10.00 usec
PL1           -4.80 dB
PL1W          13.65439701 W
SFO1          125.7703643 MHz

===== CHANNEL f2 =====
CPDPRG2       waltz16
NUC2          1H
PCPD2         80.00 usec
PL2           -4.80 dB
PL12          12.80 dB
PL13          16.00 dB
PL2W          7.58577585 W
PL12W         0.13182567 W
PL13W         0.06309573 W
SFO2          500.1320005 MHz
SI            32768
SF            125.7577890 MHz
WDW           EM
SSB           0
LB            1.00 Hz
GB            0
PC            0.30
    
```



## $\alpha,\alpha$ -DICHLOROISOXAZOLIDINONES FOR THE SYNTHESIS AND CHEMOSELECTIVE PEPTIDE LIGATION OF $\alpha$ -PEPTIDE $\alpha$ - KETOACIDS

Tetsuo Narumi<sup>1</sup> and Jeffrey W. Bode<sup>2\*</sup>

Roy and Diana Vagelos Laboratories, Department of Chemistry, University of  
Pennsylvania, Philadelphia, PA 19104, USA. bode@org.chem.ethz.ch

**Abstract** – In seeking to develop an iterative approach to the preparation of  $\alpha$ -oligopeptides by the chemoselective amide-forming coupling of  $\alpha$ -ketoacids and hydroxylamines, we have designed and synthesized novel enantiopure monomers. Key to our approach is the use of  $\alpha,\alpha$ -dichloroacids as masked  $\alpha$ -ketoacids. The preparation of these monomers, their coupling with  $\alpha$ -ketoacids, and the conversion of the  $\alpha,\alpha$ -dichloroacids to  $\alpha$ -ketoacids is described. These studies provide a first step to a conceptually unique approach to peptide synthesis that does not require activating reagents or produce chemical byproducts.

*This paper is dedicated to Professor Albert Eschenmoser on the occasion of his 85<sup>th</sup> birthday.*

### INTRODUCTION

The chemical and biogenic synthesis of  $\alpha$ -amino acids relies on the activation of the carboxylic acid moiety towards coupling with an amine nucleophile.<sup>1</sup> The success and prevalence of this paradigm has obscured the consideration of alternative approaches to iterative peptide couplings that could have advantages in chemical synthesis or a potential role in the prebiotic or exobiotic assembly of oligopeptide chains. In addressing this, we have sought to develop methods for amide bond formation that are chemically distinct from classical peptide synthesis and have recently identified the coupling of  $\alpha$ -

---

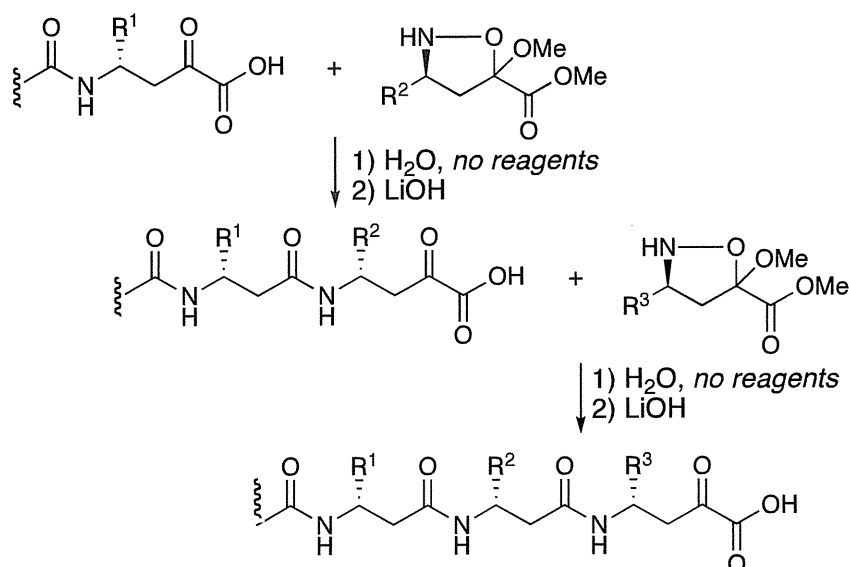
<sup>1</sup> Present address: Department of Medicinal Chemistry, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, 2-3-10 Kanda surugadai, Chiyoda-ku, Tokyo 101-0062, JAPAN

<sup>2</sup> Present address: Laboratorium für Organische Chemie, ETH-Zürich, Wolfgang Pauli Strasse 10, Zürich 8093, Switzerland.



ketoacids and hydroxylamines as a novel, chemoselective, and water-compatible approach to amide and peptide bond formation.<sup>2</sup>

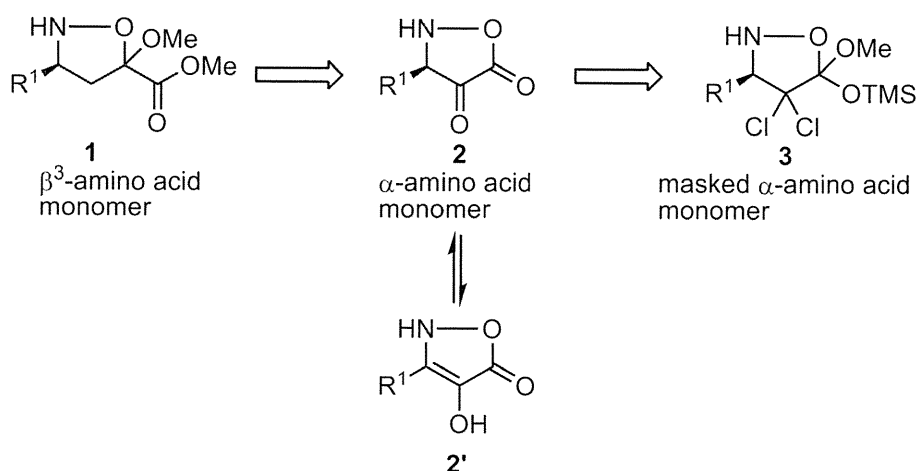
We have previously documented the application of the  $\alpha$ -ketoacid–hydroxylamine ligation (KAHA ligation) to the iterative synthesis of  $\beta^3$ -oligopeptides.<sup>3</sup> These reactions proceed in water, do not require coupling reagents, and produce only CO<sub>2</sub> and MeOH as reaction byproducts (Figure 1). Provided that suitable enantiopure monomers for the preparation of  $\alpha$ -oligopeptides could be identified, this chemistry could offer a novel and chemically orthogonal route to iterative peptide synthesis. Importantly, such an approach could obviate the problematic and wasteful coupling reagents typically employed in large excess during classical peptide synthesis.<sup>4</sup>



**Figure 1.** Iterative synthesis of  $\beta^3$ -oligopeptides by chemoselective amidation without reagents or byproducts.

The difficulty in extending our earlier work to the iterative synthesis of  $\alpha$ -oligopeptides lies in the identification and synthesis of a suitable monomer class to serve as the building blocks for the amino acid residues. These monomers must 1) cleanly afford the peptides by decarboxylative condensations with  $\alpha$ -ketoacids, 2) result in products that can be converted to the next  $\alpha$ -ketoacids following the ligation, and 3) be amenable to preparation in enantiopure form on a multigram scale. Initially, we envisioned an extension of the isoxazolidine approach we applied towards the preparation of  $\beta$ -peptides, which would require  $\alpha$ -ketolactone monomers **2**. However, such monomers are likely to be highly prone to epimerization, as such 5-membered ring heterocycles have a tendency to exist predominantly in the conjugated enol form.<sup>5</sup> After considering several alternatives, we elected to pursue the use of  $\alpha,\alpha$ -

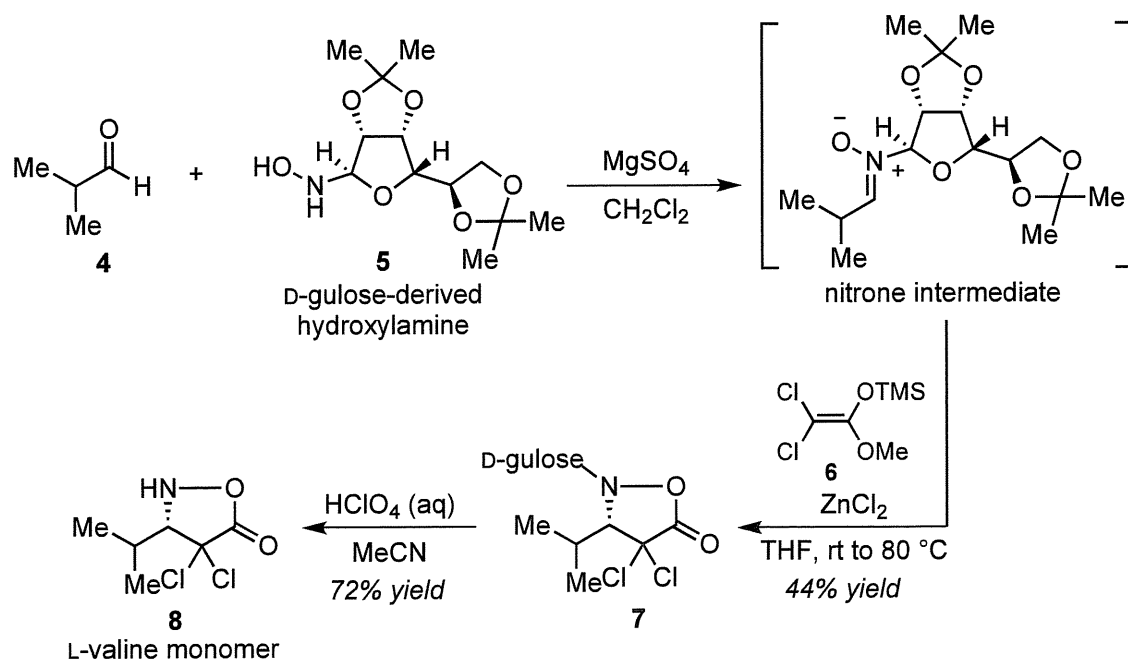
dichloroacids as masked  $\alpha$ -ketoacid surrogates (Figure 2). In this article, we describe our preliminary investigations into the preparation of enantiomerically enriched  $\alpha,\alpha$ -dichloroisoxazolidinones and their use for the iterative synthesis of  $\alpha$ -oligopeptides. These studies establish a synthetic entry into these monomers, their viability in the key amide-forming ligation reaction, and the conversion of the resulting  $\alpha,\alpha$ -dichloroacids into the corresponding  $\alpha$ -ketoacids.



**Figure 2.** Design of monomers for iterative  $\alpha$ -peptide synthesis.

## RESULTS AND DISCUSSION

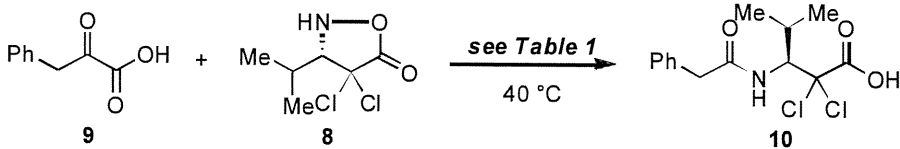
Our synthetic route to the enantiomerically enriched valine-derived dichloro monomer is illustrated in Scheme 1. After exploring several different cycloaddition routes, we selected a modification of Vasella's carbohydrate-derived nitrene as a viable synthesis of monomer **8**.<sup>6</sup> Simply heating a mixture of the nitrene, prepared from isobutyl aldehyde **4** and D-Gulose-derived hydroxylamine **5**,<sup>78</sup> with  $\alpha,\alpha$ -dichloro ketene silyl acetal **6**<sup>9</sup> containing a stoichiometric amount of  $\text{ZnCl}_2$  provided directly the  $\alpha,\alpha$ -dichloroisoxazolidin-5-ones **7** in 44% yield with high regio- and diastereoselectivity, rather than the expected  $\alpha,\alpha$ -dichloroisoxazolidine.<sup>1011</sup> Removal of the chiral auxiliary by perchloric acid-mediated hydrolysis provided L- $\alpha$ -Val monomer **8** in 72% yield. The enantiomer D- $\alpha$ -Val monomer **ent-8** was prepared from L-Gulose-derived hydroxylamine by a similar sequence.



**Scheme 1.** Preparation of L-Valine-derived  $\alpha,\alpha$ -dichloroisoxazolidinone monomer.

Although the condensation of the nitron and the silyl ketene acetal could also be applied to give isoxazolidinones corresponding to leucine, phenylalanine, and alanine-monomers, the removal of the chiral auxiliary proved problematic. We believe that this can be addressed with optimization, but have instead pressed on with our exploration of the chemistry of these heterocycles using the valine-derived monomers.

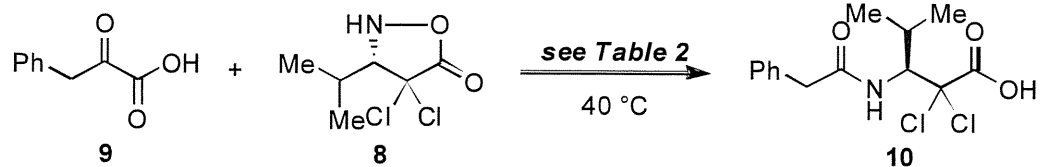
With  $\alpha,\alpha$ -dichloro-isoxazolidinone **8** in hand, the decarboxylative amide bond formation with phenylpyruvic acid (**9**) was examined (Table 1). Polar solvents such as DMF or DMSO are ideal solvents for the coupling of O-unsubstituted hydroxylamines.<sup>2</sup> On the other hand, non-polar or protic solvents including aqueous media are suitable for the coupling of isoxazolidine-based monomer.<sup>3</sup> We therefore began our studies with a screen of solvents for the amide-formation using phenylpyruvic acid (1.5 equiv) and 0.1 M monomer **8** at 40 °C for 12 h. Non-polar solvents such as  $\text{CH}_2\text{Cl}_2$ , toluene and THF were unproductive (entries 1-3). Polar solvents, including DMF and DMSO, gave the desired amides in low conversion along with significant amounts of unidentified products (entries 4 and 5), probably due to the decomposition of the starting material. A 1:1 mixture of  $t$ BuOH and water gave the desired amide **10** cleanly, albeit with low conversion (entry 6). Reaction with higher substrate concentration (0.2 M) gave **10** with improved conversion (entry 7), but further increases in concentration did not improve the outcome (entry 8). Additional optimization of these conditions revealed that MeOH or EtOH were superior cosolvents (entries 9–11). Alcohol solvents alone, however, did not afford amide products (entry 12).

**Table 1.** Conditions for amide-forming reactions of  $\alpha$ -ketoacids and isoxazolidinone **8**.

entry	conditions <sup>a</sup>	conversion (%) <sup>b</sup>
1	CH <sub>2</sub> Cl <sub>2</sub> (0.1 M)	nr
2	toluene (0.1 M)	nr
3	THF (0.1 M)	nr
4	DMF (0.1 M)	27 <sup>c</sup>
5	DMSO (0.1 M)	25 <sup>c</sup>
6	1:1 H <sub>2</sub> O/ <sup>t</sup> BuOH (0.1 M)	12
7	1:1 H <sub>2</sub> O/ <sup>t</sup> BuOH (0.2 M)	31
8	1:1 H <sub>2</sub> O/ <sup>t</sup> BuOH (0.4 M)	26
9	1:1 H <sub>2</sub> O/MeOH (0.2 M)	77
10	1:1 H <sub>2</sub> O/EtOH (0.2 M)	75
11	1:1 H <sub>2</sub> O/ <sup>i</sup> PrOH (0.2 M)	55
12	MeOH (0.2 M)	trace

<sup>a</sup> All reactions were carried out using Val monomer **8** and phenylpyruvic acid **9** (1.5 equiv) at 40 °C for 12 h. <sup>b</sup> determined by <sup>1</sup>H-NMR. <sup>c</sup> A significant amount of unidentified products was detected.

Having identified aqueous alcohol as the preferred solvent for amide-bond formation, we performed a second level optimization of the reaction pH (Table 2). The ligations occurred over a wide pH range, with slightly acidic oxalic acid buffers offering the best results.

**Table 2.** Optimization of pH for amide-forming ligations.

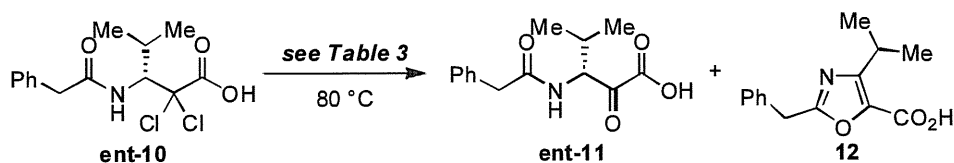
entry	conditions <sup>a</sup>	conversion (%) <sup>b</sup>
1	1:1 H <sub>2</sub> O/MeOH	77
2	1:1 pH 3.4 Ac buffer/MeOH	74
3	1:1 pH 4.6 Ac buffer/MeOH	68
4	1:1 pH 5.3 Ac buffer/MeOH	77
5	1:1 pH 5.3 citric buffer/MeOH	66
6	1:1 pH 6.2 citric buffer/MeOH	78
7	1:1 pH 5.3 oxalic buffer/MeOH	80
8	1:1 pH 6.1 oxalic buffer/MeOH	90
9	1:1 pH 7.4 phosphate buffer/MeOH	22

<sup>a</sup> All reactions were carried out using Val monomer **8** and phenylpyruvic acid **9** (1.5 equiv) in 0.2 M solution at 40 °C for 12 h. <sup>b</sup> determined by <sup>1</sup>H-NMR.

The key underlying concept to the proposed iterative synthesis of  $\alpha$ -oligopeptides is the use of the  $\alpha,\alpha$ -dichloro carboxylic acid as a masked  $\alpha$ -ketoacids. Although the conversions of dihaloacids and related compounds to  $\alpha$ -ketoacids are rare, several encouraging examples were known<sup>12</sup> including a thorough study from a group at Bristol-Myers Squibb that implicated the formation of an  $\alpha$ -lactone in the hydrolysis.<sup>13</sup> It was unclear, however, if any of these methods would be compatible with the proximal amide functionality and the epimerizable  $\alpha$ -stereocenter.

Working from the BMS precedent for conversion of  $\alpha,\alpha$ -dichloroacids **10** to the  $\alpha$ -ketoacids **11**, we began our investigations with base promoted hydrolysis (Table 3). Strong bases including LiOH (entry 1), K<sub>3</sub>PO<sub>4</sub> (entry 2), and Na<sub>2</sub>CO<sub>3</sub> (entry 3) afforded exclusively undesired oxazole **12**. Success was obtained with weaker bases (entries 4–8) and several conditions afforded the  $\alpha$ -ketoacids as the major product. Although the oxazole byproduct could not be completely excluded, acceptable yields could be obtained with potassium bases including potassium oxalate at 80 °C (entry 8).

**Table 3.** Conditions for conversion of  $\alpha,\alpha$ -dichloroacid **10** to  $\alpha$ -ketoacid **11**.

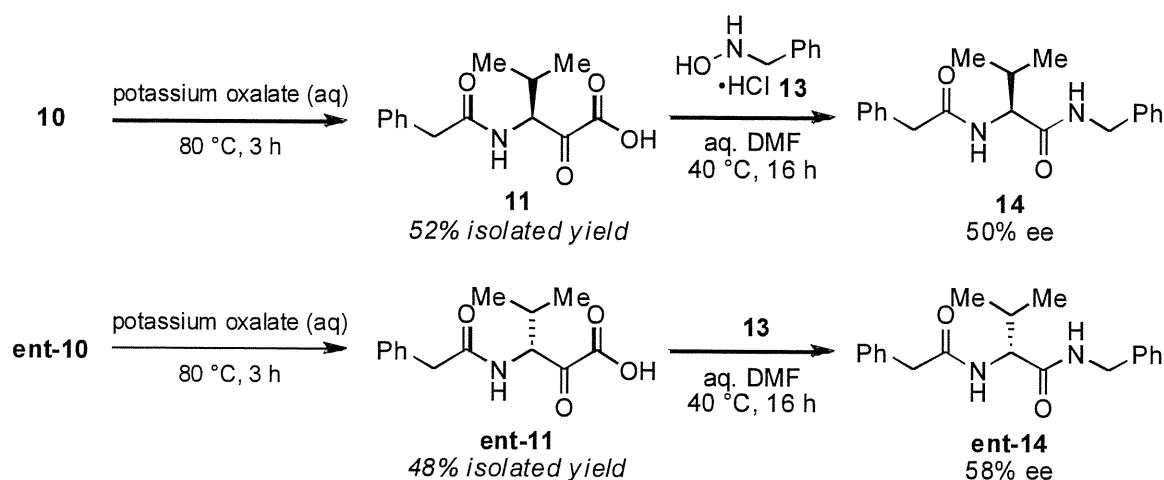


entry	condition <sup>a</sup> (1.0 M)	conv. to <b>11</b> (%) <sup>b</sup>	conv. to <b>12</b> (%) <sup>b</sup>
1	LiOH	nd	73
2	K <sub>3</sub> PO <sub>4</sub>	nd	>90
3	Na <sub>2</sub> CO <sub>3</sub>	nd	40
4	AcONa	43	39
5	Na <sub>2</sub> HPO <sub>4</sub>	26	26
6	sodium citrate	44	33
7	K <sub>2</sub> HPO <sub>4</sub>	60	22
8	potassium oxalate	62	33

<sup>a</sup> All reactions were carried out using  $\alpha,\alpha$ -dichloroacid **10** in 1.0 M aqueous solution at 80 °C for 3 h. <sup>b</sup> determined by RP-HPLC.

At this juncture, we sought to address the critical questions of the stereochemical integrity of the  $\alpha$ -stereocenter under the basic conditions necessary for the unmasking of the  $\alpha$ -ketoacids. In our experience, which is consistent with the chemical literature,<sup>14</sup>  $\alpha$ -ketoacids are configurationally stable in organic solvents and in the presence of aqueous acid, but are prone to racemization in the presence of base.<sup>15</sup> They are generally stable under the ligation conditions.

$\alpha,\alpha$ -Dichloroacid **10** and its enantiomer **ent-10**, prepared from the L-gulose-derived chiral auxiliary, were each hydrolyzed with 1.0 M potassium oxalate at 80 °C for 3 h and the resulting  $\alpha$ -ketoacids isolated in 48–52% yield by preparative HPLC (Scheme 2). We were not able to assay the enantiopurity of  $\alpha$ -ketoacid **11** directly and therefore elected to perform a second ligation prior to analysis. Each enantiomer of valine-derived  $\alpha$ -ketoacid **11** was subjected to ligation with benzyloxyamine **13** in aqueous DMF under unoptimized conditions and the resulting amides analyzed by SFC on a AD-H column. Unfortunately, this study revealed significant epimerization of the valine residue, presumably due to the basic conditions employed in the hydrolysis of the  $\alpha,\alpha$ -dichloroacid.



**Scheme 2.** Investigation of epimerization during hydrolysis of  $\alpha,\alpha$ -dichloroacid **10**.

In preliminary work, we have prepared dichloroisoxazoline monomers corresponding to other amino acid residues including L-leucine and L-phenylalanine and found that these examples are less prone to racemization during hydrolysis, suggesting that improvements to these protocols are possible. Further efforts to improve the hydrolysis conditions and the preparation of the monomers may provide an alternative route to the iterative synthesis of  $\alpha$ -peptides that does not require coupling reagents or protecting groups and which operates under aqueous conditions

## ACKNOWLEDGMENTS

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## EXPERIMENTAL

**General Methods.** All reactions utilizing air- or moisture-sensitive reagents were performed in dried

glassware under an atmosphere of dry nitrogen. Thin layer chromatography (TLC) was performed on EMD precoated plates (silica gel 60 F254, Art 5715) and were visualized by fluorescence quenching under UV light and by staining with phosphomolybdic acid and/or *p*-anisaldehyde, respectively. For flash column chromatography, EMD Silica Gel 60 (230–400 Mesh) and Silica Gel 60 N (Kanto Chemical Co., Inc.) were employed. <sup>1</sup>H NMR (500 MHz) and <sup>13</sup>C NMR (125 MHz) were measured on a Bruker Avance AVII-500 spectrometer with a CryoProbe. Chemical shifts are expressed in parts per million (ppm) with respect to the residual solvent peak. Coupling constants are reported as Hertz (Hz), signal shapes and splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Infrared (IR) spectra were recorded on a SHIMADZU IR Prestige-21 FTIR-8400S and are reported as wavenumber (cm<sup>-1</sup>). Low- and high-resolution mass spectra were recorded on a Bruker Daltonics microTOF-2focus in the positive and negative detection mode. Optical rotations were measured on JASCO DIP-370 polarimeter operating at the sodium D line with a 100 mm path length cell, and were reported as follows:  $[\alpha]_D^{25}$  (concentration (g:100 mL), solvent).

**HPLC Conditions.** For analytical HPLC, a Cosmosil 5C<sub>18</sub>-ARII column (4.6 x 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of MeCN containing 0.1% (v/v) TFA at a flow rate of 1 cm<sup>3</sup> min<sup>-1</sup> on a LaChrom Elite HTA system (Hitachi High-Technologies corporation, Ltd., Tokyo, Japan) and JASCO PU-2086 plus (JASCO corporation, Ltd., Tokyo, Japan), and eluting products were detected by UV at 220 nm. Preparative HPLC was performed using a Cosmosil 5C<sub>18</sub>-AR II column (20 x 250 mm, Nacalai Tesque, Inc.) on a JASCO PU-2087 plus and PU-2089 plus (JASCO corporation, Ltd., Tokyo, Japan) in a suitable gradient mode of MeCN solution containing 0.1% (v/v) TFA at a flow rate of 7-10 cm<sup>3</sup> min<sup>-1</sup>.

**(2,2-Dichloro-1-methoxyvinloxy)trimethylsilane 6.** To a suspension of activated Zn dust (784.8 mg, 12.0 mmol) in THF (10 mL) was added dropwise a solution of Cl<sub>3</sub>CCO<sub>2</sub>Me (0.953 mL, 8.0 mmol) and TMSCl (1.23 mL, 9.6 mmol) at ambient temperature (< 40 °C). After stirring at room temperature overnight, the reaction mixture had changed to a clear cream-colored suspension containing the excess Zn dust. This mixture was used in the next step without further purification.

**(S)-4,4-Dichloro-2-((3aR,4R,6S,6aR)-6-((R)-2,2-dimethyl-1,3-dioxolan-4-yl)-2,2-dimethyltetrahydrofuro[3,4-d][1,3]dioxol-4-yl)-3-isopropylisoxazolidin-5-one 7.** To a solution of D-gulose derived chiral auxiliary **1**<sup>16</sup> (562.6 mg, 2.0 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added isobutylaldehyde (272.4 μL, 3.0 mmol) in the presence of MgSO<sub>4</sub> (1 g). After stirring at rt for 3 h, the reaction mixture was filtered to remove MgSO<sub>4</sub>. Concentration under reduced pressure gave the nitrone as a white solid, which was used in the

next step without further purification. To a solution of the nitron in THF (10 mL) was added a solution of  $\alpha,\alpha$ -dichloroketene silyl acetal **6** (0.80 M, 8.0 mmol) containing a stoichiometric amount of  $\text{ZnCl}_2$  in THF (10 mL), from which the excess Zn dust was removed with a PTFE filter (0.45  $\mu\text{m}$ ). After stirring at rt for 30 min, the mixture was refluxed for 6 h. After cooling to room temperature, the reaction mixture was poured into ice-cold saturated aqueous  $\text{NaHCO}_3$  and extracted with EtOAc. The extract was washed with brine and dried over  $\text{MgSO}_4$ . Concentration under reduced pressure followed by flash chromatography over silica gel with hexanes-EtOAc (9:1) gave **7** (382.7 mg, 44% yield) as a colorless oil:  $[\alpha]_D^{25} +3.74$  (c 0.710,  $\text{CHCl}_3$ );  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.39 (s, 1H), 4.96 (d,  $J = 6.0$  Hz, 1H), 4.77 (dd,  $J = 6.0, 4.0$  Hz, 1H), 4.39 (dt,  $J = 8.5, 6.5$  Hz, 1H), 4.32 (d,  $J = 3.0$  Hz, 1H), 4.23 (dd,  $J = 9.0, 7.0$  Hz, 1H), 4.15 (dd,  $J = 8.5, 4.0$  Hz, 1H), 3.76 (dd,  $J = 8.5, 6.5$  Hz, 1H), 2.43-2.34 (m, 1H), 1.46 (s, 3H), 1.39 (s, 3H), 1.38 (s, 3H), 1.31 (s, 3H), 1.06 (d,  $J = 7.0$  Hz, 3H), 0.882 (d,  $J = 6.5$  Hz, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  168.5, 113.4, 109.9, 97.8, 85.6, 84.1, 80.2, 78.4, 75.4, 74.4, 65.9, 31.2, 26.5, 26.0, 25.1, 24.7, 21.0, 15.7; IR (KBr)  $\nu$  2981, 1807, 1209, 1080, 979, 948, 894, 864, 848, 759  $\text{cm}^{-1}$ ; HRMS (ESI),  $m/z$  calcd for  $\text{C}_{18}\text{H}_{28}\text{Cl}_2\text{NO}_7$   $[\text{M}+\text{H}]^+$  440.1243 and 442.1213, found 440.1245 and 442.1214.

**(S)-4,4-Dichloro-3-isopropylisoxazolidin-5-one 8 (L-Val monomer)**. To a solution of D-gulosyl L-Val monomer **7** (102.8 mg, 0.233 mmol) in MeCN (2.3 mL) was added 60%  $\text{HClO}_4$  (116.7  $\mu\text{L}$ , 0.700 mmol), and the mixture was stirred at rt for 1.5 h. After being diluted with EtOAc, the mixture was washed with saturated aqueous  $\text{NaHCO}_3$  and brine, and dried over  $\text{MgSO}_4$ . Concentration under reduced pressure followed by flash chromatography hexanes-EtOAc (9:1) gave **8** (33.3 mg, 72% yield) as a colorless oil:  $[\alpha]_D^{25} -3.18$  (c 1.27,  $\text{CHCl}_3$ );  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  6.70 (br, 1H), 3.69 (dd,  $J = 12.3, 8.9$  Hz, 1H), 2.01-2.20 (br, 1H), 1.24 (d,  $J = 6.7$  Hz, 3H), 1.24 (d,  $J = 6.7$  Hz, 1H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  170.0, 79.5, 76.0, 28.2, 19.0; IR (KBr)  $\nu$  3431, 3226, 1811, 1197, 759  $\text{cm}^{-1}$ ; HRMS (ESI),  $m/z$  calcd for  $\text{C}_6\text{H}_{10}\text{Cl}_2\text{NO}_2$   $[\text{M}+\text{H}]^+$  198.0089 and 200.0059, found 198.0082 and 200.0056.

**(S)-2,2-Dichloro-4-methyl-3-(2-phenylacetamido)pentanoic acid 10**. To a solution of L-Val monomer **8** (61.4 mg, 0.310 mmol) in a 1:1 mixture (1.55 mL) of MeOH/oxalic buffer (pH 6.1) was added phenylpyruvic acid (76.4 mg, 0.465 mmol). After being stirred at 40  $^\circ\text{C}$  for 41 h, purification by preparative HPLC (Gradient: 0 min, 30% MeCN in  $\text{H}_2\text{O}$ ; 90 min, 60% MeCN in  $\text{H}_2\text{O}$ ) followed by lyophilization afforded compound **10** (63.4 mg, 64% yield) as colorless powder:  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.40-7.28 (m, 5H), 5.97 (d,  $J = 10.5$  Hz, 1H), 4.82 (dd,  $J = 10.5, 3.5$  Hz, 1H), 3.82 (d,  $J = 16.5$ , 1H), 3.74 (d,  $J = 16.5$ , 1H), 2.37-2.27 (m, 1H), 0.956 (d,  $J = 7.0$ , 3H), 0.745 (d,  $J = 6.5$ , 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  173.6, 166.5, 133.4, 129.7, 129.4, 128.0, 87.1, 60.7, 43.2, 29.4, 22.0, 17.2; HRMS (ESI),  $m/z$  calcd for  $\text{C}_{14}\text{H}_{18}\text{Cl}_2\text{NO}_3$   $[\text{M}+\text{H}]^+$  318.0664 and 320.0634, found 318.0653 and 320.0630.

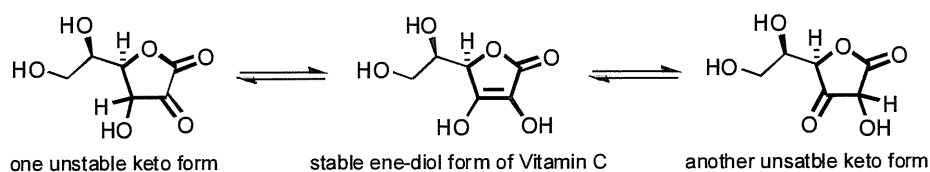


**(S)-4-Methyl-2-oxo-3-(2-phenylacetamido)pentanoic acid 11.** Dichloro-isoxazolidinone **10** (55.6 mg, 0.175 mmol) was treated with 1.0 M aqueous solution of potassium oxalate (3.5 mL) at 80 °C for 3 h. After cooling to room temperature, purification by preparative HPLC (Gradient: 0 min, 25% MeCN in H<sub>2</sub>O; 90 min, 55% MeCN in H<sub>2</sub>O) followed by lyophilization afforded **11** (23.8 mg, 52% yield) as a colorless oil: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.41-7.27 (m, 5H), 6.14 (br, 1H), 5.03 (br, 1H), 3.69 (s, 2H), 2.30-2.23 (m, 1H), 0.924 (d, *J* = 7.0 Hz, 3H), 0.683 (d, *J* = 7.0 Hz, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 193.1, 173.3, 160.5, 133.5, 129.4, 129.3, 127.9, 60.0, 43.0, 29.6, 19.6, 17.0; IR (KBr) ν 3388, 1724, 1647, 1531, 1215, 761 cm<sup>-1</sup>; HRMS (ESI), *m/z* calcd for C<sub>14</sub>H<sub>18</sub>NO<sub>4</sub> [M+H]<sup>+</sup> 264.1236, found 264.1239.

**2-Benzyl-4-isopropylloxazole-5-carboxylic acid 12.** Dichloro-isoxazolidine **10** (55.6 mg, 0.175 mmol) was converted into the title compound **12** (11.2 mg, 26% yield) as colorless freeze-dried powder: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.34-7.27 (m, 5H), 4.17 (s, 2H), 3.67-3.51 (m, 1H), 1.27 (d, *J* = 6.5, 6H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 165.6, 162.6, 157.6, 135.39, 134.4, 128.9, 128.8, 127.3, 34.8, 26.0, 21.3; IR (KBr) ν 3412, 1612, 758 cm<sup>-1</sup>; HRMS (ESI), *m/z* calcd for C<sub>14</sub>H<sub>16</sub>NO<sub>3</sub> [M+H]<sup>+</sup> 246.1130, found 246.1127. This is the side product of the previous reaction.

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# Fluorescent-Responsive Synthetic C1b Domains of Protein Kinase C $\delta$ as Reporters of Specific High-Affinity Ligand Binding

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Protein kinase C (PKC) is a critical cell signaling pathway involved in many disorders such as cancer and Alzheimer-type dementia. To date, evaluation of PKC ligand binding affinity has been performed by competitive studies against radiolabeled probes that are problematic for high-throughput screening. In the present study, we have developed a fluorescent-based binding assay system for identifying ligands that target the PKC ligand binding domain (C1 domain). An environmentally sensitive fluorescent dye (solvatochromic fluorophore), which has been used in multiple applications to assess protein-binding interactions, was inserted in proximity to the binding pocket of a novel PKC $\delta$  C1b domain. These resultant fluorescent-labeled  $\delta$ C1b domain analogues underwent a significant change in fluorescent intensity upon ligand binding, and we further demonstrate that the fluorescent  $\delta$ C1b domain analogues can be used to evaluate ligand binding affinity.

## INTRODUCTION

Protein kinase C (PKC) is a family of serine/threonine protein kinases comprising 11 isozymes divided into three subclasses, termed conventional ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), novel ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ), and atypical ( $\zeta$ ,  $\lambda$ ,  $\iota$ ). Their classification is based on their essential structures and affinities for regulatory factors such as diacylglycerol (DAG) and calcium that bind to the C1 and C2 domains, respectively, of PKC. PKC plays a pivotal role in physiological responses to growth factors, oxidative stress, and tumor promoters (phorbol esters). These responses regulate numerous cellular processes (1, 2), including proliferation (3), differentiation (4), migration (5), and apoptosis (6, 7). The extensive involvement of PKC in both normal physiology and in numerous disorders has caused PKC to emerge as an important therapeutic target (8–10). Since PKC activation is regulated through the binding of ligands to its C1 domains, development of useful ligands targeted to the C1 domains has been of intense interest for medicinal chemists. Various synthetic PKC ligands based on  $\gamma$ -lactone templates have been developed and evaluated (11–13). On the other hand, since structures of many of the potent, naturally occurring PKC ligands such as the phorbol esters are highly complex, it has been difficult to extensively probe their structure–activity relationships. Much opportunity therefore remains for the development of ligands optimized for isozyme selectivity or other properties. Fluorescent-based methods possess many advantages for high-throughput screening. Especially, utilization of environmentally sensitive fluorophores is suitable for high-throughput techniques because washing steps are not generally required. Fluorophores often respond to the environmental changes in hydrophobic/hydrophilic states associated with the conformational changes of proteins accompanying ligand binding. Several screening methods based on fluorescent-modified

peptides, e.g., an IP<sub>3</sub> sensor, have been developed to date (14–17). In this study, fluorescent-labeled C1b domains of PKC $\delta$  utilizing a solvatochromic dye as a sensor of ligand binding were designed and synthesized as efficient screening tools to evaluate ligand binding and to explore novel PKC pharmacophores.

## EXPERIMENTAL PROCEDURES

**General Methods.** For chromatography, Wakogel C-200 (Wako Pure Chemical Industries, Ltd.) was employed. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded using a Bruker Ultrashield Plus Avance 400 spectrometer. Relative chemical shifts were reported in  $\delta$  (ppm) in DMSO-*d*<sub>6</sub> or in CDCl<sub>3</sub> with tetramethylsilane as an internal standard. Low- and high-resolution mass spectra were recorded on a JMS-T1000LC AccuTOF and Bruker Daltonics microTOF-2focus. RP-HPLC was performed with linear gradients of acetonitrile and H<sub>2</sub>O containing 0.1% (v/v) TFA (column: Cosmosil <sub>5</sub>C<sub>18</sub> AR-II (4.6 × 250 mm) for analytical runs, Cosmosil <sub>5</sub>C<sub>18</sub> AR-II (20 × 250 mm) for preparative runs. UV absorbance spectra were recorded on a Jasco V-650 spectrophotometer using a 1.0 cm path length quartz cuvette. Fluorescent spectra were recorded on a Jasco FP-6600 spectrofluorometer using a 1.0 cm path length quartz cuvette. Measurements of fluorescent intensity on 96-well plates were performed on a Wallac ARVO MX (Perkin-Elmer).

**Peptide Synthesis.** The protected peptide of  $\delta$ C1b(247–281) was manually constructed on a Novasyn TGR resin (0.25 mmol/g) by standard Fmoc-based solid phase peptide synthesis (SPPS). Fmoc-protected amino acid derivatives (5 equiv) were successively condensed using 1,3-diisopropylcarbodiimide (DIPCI) (5 equiv) in the presence of 1-hydroxybenzotriazole·H<sub>2</sub>O (HOBt·H<sub>2</sub>O) (5 equiv) in DMF (2 mL) (90 min treatment). The following side-chain protecting groups were used: Boc for Lys; Pbf for Arg; OBU<sup>t</sup> for Asp; Trt for Asn, Cys, and His; Bu<sup>t</sup> for Ser, Thr, and Tyr. The Fmoc group was deprotected with 20% (v/v) piperidine in DMF (2 mL) for 15 min. The resulting protected peptide was cleaved from the resin and deprotected with TFA-thioanisole-*m*-cresol-triisopropylsilane (TIS) (89:7.5:2.5:1, v/v) (90 min treatment). Deprotected peptides were

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washed with cold Et<sub>2</sub>O three times. The product was then purified by RP-HPLC. The protected peptides of dansyl-labeled  $\delta$ C1b(221–246) were manually elongated on an Fmoc-His(Trt)-Trt(2-Cl)-resin (0.42 mmol/g) by Fmoc-based SPPS as in the synthesis of  $\delta$ C1b(247–281). The following side-chain protecting groups were used: Boc for Lys; Pbf for Arg; OBU<sup>t</sup> for Asp, Glu; Trt for Asn, Cys, Gln, and His; Bu<sup>t</sup> for Ser, Thr, and Tyr. At the dansyl-labeled position, Fmoc-Lys(DnsGly)-OH was used. The resulting protected peptides were cleaved from the resin with trifluoroethanol (TFE)-AcOH-DCM (1:1:3, v/v) (2 h treatment), followed by thioesterification. Deprotection was performed as in the synthesis of  $\delta$ C1b(247–281). The product was then purified by RP-HPLC. Mass data and chemical yields of these peptides are described in Supporting Information.

**Thioesterification.** Thioesterification was performed with ethyl mercaptopropionate (20 equiv), HOBT·H<sub>2</sub>O (10 equiv), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI)·HCl (10 equiv) in DMF (1 mL) (–20 °C, overnight). DMF was removed by evaporation, and the crude products were washed with H<sub>2</sub>O.

**Native Chemical Ligation.** Dansyl-labeled  $\delta$ C1b(221–246) (1.8 mg, 0.5  $\mu$ mol) and the  $\delta$ C1b(247–281) (1.9 mg, 0.5  $\mu$ mol) were dissolved in 500  $\mu$ L of 100 mM phosphate buffer (pH 8.5) containing 6 M guanidine hydrochloride (Gn·HCl) containing 2 mM EDTA and tris(2-carboxyethyl)phosphine hydrochloride (TCEP·HCl) (1.4 mg, 5  $\mu$ mol). Thiophenol (15 mL, 3%) was then added to the mixture. The ligation reaction was performed at 37 °C under an N<sub>2</sub> atmosphere. Progress of the ligation reaction was monitored by RP-HPLC (gradient: 25–45% of acetonitrile/0.1% TFA against H<sub>2</sub>O/0.1% TFA). The product was subjected to gel filtration with Sephadex G-10 and then purified by RP-HPLC. Mass data of these peptides are described in Supporting Information.

**Folding of  $\delta$ C1b Domains.** Purified peptides were dissolved in 50 mM Tris·HCl (pH 7.4) with 5 mM DTT, incubated for 15 min at 30 °C, and then stored at –20 °C with 20% glycerol. The peptide solution was dialyzed against 50 mM Tris·HCl (pH 7.4) containing 150 mM NaCl, 1 mM DTT, and 0.1 mM ZnCl<sub>2</sub> using a Slide-A-Lyzer Dialysis Cassette 2000 MWCO (Thermo Scientific).

**[<sup>3</sup>H]PDBu Binding Assay.** [<sup>3</sup>H]PDBu binding to the  $\delta$ C1b domains was measured using the poly(ethylene glycol) precipitation assay as described previously (18, 19) with minor modifications. To determine the dissociation constants ( $K_d$ ) and numbers of binding sites ( $B_{max}$ ) for the dansyl-labeled  $\delta$ C1b domains, saturation curves with increasing concentrations of [<sup>3</sup>H]PDBu were obtained in triplicate. 250  $\mu$ L of the assay mixture contained 50 mM Tris·HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EGTA), 0.1 mg/mL phosphatidylserine, 5 mg/mL bovine immunoglobulin G, variable concentrations of [<sup>3</sup>H]PDBu, and, for those tubes used to determine nonspecific binding, an excess of nonradioactive PDBu. After addition of peptides stored in 0.015% Triton X-100, binding was carried out at 18 °C for 10 min. Samples were incubated on ice for 10 min. 200  $\mu$ L of 35% poly(ethylene glycol) in 50 mM Tris·HCl (pH 7.4) was added, and the samples were incubated on ice for an additional 10 min. The tubes were centrifuged at 4 °C (12 200 rpm, 15 min), and a 100  $\mu$ L aliquot of each supernatant was then transferred to a scintillation vial for determination of the amount of the free [<sup>3</sup>H]PDBu. After the remaining supernatant was aspirated off, the bottom of each centrifuge tube was cut off just above the pellet and transferred to a scintillation vial for the determination of the amount of the total bound [<sup>3</sup>H]PDBu. Dissociation constants ( $K_d$ ) were calculated by Scatchard analysis.

**CD Measurement.** CD spectra were recorded on a Jasco J-720 spectropolarimeter at 25 °C. The measurements were

performed at 0.1 nm spectral resolution using a 0.5 cm path length quartz cuvette. Each spectrum represents the average of 40 scans, and the scan rate was 100 nm/min. Measurements were performed in a Tris·HCl (pH 7.4) buffer containing 1 mM DTT and 150 mM NaCl.

**Fluorescence Measurement.** Ligand titration was performed in 1 mL of dialysis buffer containing 0.5  $\mu$ M dialyzed dansyl-labeled  $\delta$ C1b domain and 5  $\mu$ g/mL phosphatidylserine at 25 °C. After each addition of ligands, the mixture was incubated at the same temperature for 10 min. Fluorescent emission spectra ( $\lambda_{ex}$  = 330 nm; slit width: 20 nm for excitation, 40 nm for emission) were obtained throughout the addition of ligands. For experiments using 96-cell plates, Dansyl-labeled  $\delta$ C1b domain solution (dialysis buffer containing 0.5  $\mu$ M dialyzed peptides and 5  $\mu$ g/mL phosphatidylserine) was prepared and incubated for 10 min at room temperature. Fluorescence of dansyl-labeled  $\delta$ C1b domain was measured using an excitation filter of 355 nm (half-width: 40 nm) and an emission filter of 460 nm (half-width: 25 nm), respectively.

**Molecular Modeling.** Molecular modeling was performed using Sybyl 7.1 (Tripos Inc., St. Louis, MO). Predictive models of dansyl-labeled  $\delta$ C1b domain analogues were built by substitution of Lys(Dns-Gly) for residues Tyr238, Ser240, or Thr242 that were contained in the crystal structure of  $\delta$ C1b domain (PDB 1PTR) (20). Energy-minimization was performed on the Lys(Dns-Gly) moiety using the Tripos force field and Gasteiger-Huckel charge parameters.

## RESULTS AND DISCUSSION

**Design of Fluorescent-Labeled PKC $\delta$  C1b Domains.** Residues 221–281 of PKC $\delta$  forming the C1b domain represent the starting sequence we used for modification. To identify the optimal amino acid position for fluorescent labeling, Tyr238, Ser240, and Thr242 were selected for evaluation. Our choice was based on the following rationale: First, these residues are located at the edge of the binding pocket of the C1b domain as shown by the structure of  $\delta$ C1b-phorbol ester complex (20). Second, site-directed mutagenesis had shown that Ser240 was not necessary for the phorbol ester binding and that the  $\delta$ C1b domain mutants T242G, T242S, and T242 V had only minimal effects on the binding affinity of PDBu (2.1-, 1.1-, and 3.2-fold, respectively) (21). Third, replacement of Tyr by Gly in position 238 reduced binding affinity of [<sup>3</sup>H]PDBu by 60-fold, but maintained nanomolar affinity ( $K_d$  = 48  $\pm$  3.0 nM), which might be sufficient for the detection of PKC ligands (21). A chemically modified lysine was utilized for fluorescent labeling. For this study, a dansyl group was adopted because of its small molecular size and larger Stokes shift compared to NBD (22). Since the flexibility of the dansyl moiety might contribute to its sensitivity to ligand binding to the  $\delta$ C1b domain, glycine was incorporated between the  $\epsilon$ -amino group of lysine and the dansyl moiety as a linker to construct Lys(Dns-Gly) (Supporting Information Scheme S1). Three  $\delta$ C1b domain analogues, in which Lys(Dns-Gly) was substituted for Tyr238, Ser240, or Thr242, were designed and designated as Y238K(DnsG), S240K(DnsG), and T242K(DnsG), respectively. Predictive structural models of the dansyl-labeled  $\delta$ C1b domains were constructed based on the crystal structure of the  $\delta$ C1b domain (PDB entry 1PTR) utilizing Sybyl 7.1 (Figure 1). The models showed that the dansyl moieties of S240K(DnsG) and T242K(DnsG) are located outside the binding pocket, whereas the dansyl moiety of Y238K(DnsG) was located inside.

**Synthesis of Dansyl-Labeled  $\delta$ C1b Domain Analogues.** Fmoc-protected Lys(Dns-Gly) (4) was synthesized as described in Supporting Information Scheme S1.  $\delta$ C1b domain analogues were synthesized based on the standard Fmoc solid-phase peptide synthesis (SPPS) (23). For an efficient synthesis of