

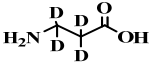
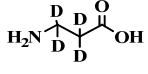
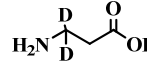
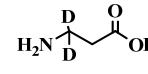
(d, J = 6.7 Hz, 3H); $^{13}\text{C-NMR}$ (D_2O , 500 MHz) δ = 177.0, 167.9, 67.7, 41.4, 36.9, 36.0, 33.1, 25.1, 22.8, 20.4; MS (M+H) $^+$ = 231.10, Cal. (M+H) $^+$ = 231.16

Compound 4

Compound 3 (115 mg, 0.50 mmol) was added to the mixture of NHS (59 mg, 0.51 mmol) and DCC (113 mg, 0.55 mmol) in 10 mL DCM. The reaction mixture was stirred for 2 days at room temperature and insoluble solid was filtered out. After the solvent was evaporated, the residue was recrystallized from diisopropyl ether to afford compound 4 (106 mg, 65% yield) as white solid.

$^1\text{H-NMR}$ (CDCl_3 , 500 MHz) δ = 3.61 (m, 1H), 3.27 (t, J = 6.5 Hz, 2H), 2.82 (t, J = 6.3 Hz, 2H), 2.66 (s, 6H), 2.61 (s, 4H), 1.74-1.61 (m, 2H), 1.43-1.37 (m, 1H), 0.94 (d, J = 6.7 Hz, 3H), 0.92 (d, J = 6.7 Hz, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 500 MHz) δ = 174.0, 169.1, 168.0, 66.8, 41.8, 36.4, 34.8, 31.4, 25.6, 25.0, 23.5, 21.7. MS (M+H) $^+$ = 328.11, Cal. (M+H) $^+$ = 328.18.

Table 1. Isotope-labeled starting materials for DiART reagents

	DiART-114	DiART-115	DiART-116	DiART-117	DiART-118	DiART-119
Leu-OH	1- ^{13}C	^{15}N	1- ^{13}C	^{15}N	1- ^{13}C	^{15}N
HCHO	unlabeled	unlabeled	unlabeled	unlabeled	DCDO	DCDO
NaCNBH ₃	unlabeled	unlabeled	NaCNBD ₃	NaCNBD ₃	unlabeled	unlabeled
β -Ala-OH					unlabeled	unlabeled

1- ^{13}C and ^{15}N labeled leucine were purchased from Cambridge Isotope Laboratory, Inc. DCDO and NaCNBD₃ were purchased from Sigma. D₄- β -alanine was purchased from C/D/N Isotopes Inc. D₂- β -alanine was synthesized in house⁴.

2. HPLC of DiART-labeled phenylalanine

Six different amount of phenylalanine were reacted with each excess DiART reagent in a mixture (NaHCO_3 , 50 mM, and 1,4-dioxane, 50%), respectively, mixed, and then purified with C₁₈-HPLC (Buffer A: water, 0.1% TFA; Buffer B: acetonitrile, 0.1% TFA; gradient: 0-6 mins 10% B, 6-8 mins 10% to 20% B, 8-48 mins 20% B to 60% B; flow rate: 1 mL/min) (see Figure 1 in this supporting information). The purified product was re-injected into HPLC using the same program. A single broad peak of the product was observed. Three time fractions of this peak were collected and analyzed with MALDI-MS/MS (see Figure 2a in paper).

3. Protein quantitation with DiART reagents and Mascot software

3.1 Detailed procedure

BSA (20 μg), bovine catalase (20 μg), and chicken ovalbumin (10 μg) were dissolved in 100 μL denaturing/reducing solution (8 M urea, 50 mM Sodium borate buffer, pH = 8.3, 5 mM TCEP) and incubated at 37 $^\circ\text{C}$ for 30 min. Then, 20 mM 2-bromoacetamide was added to alkylate free cysteine residues. The proteins were

precipitated with acetone, dissolved again in 100 μ L buffer (200 mM Sodium borate buffer, pH = 8.3, 0.8 M urea), and then digested with trypsin (10 μ g) at 37 °C overnight. Six fractions of samples (10 μ L) were mixed with 20 μ L DiART reagents (2 mg/mL in ethanol), respectively and the reaction was incubated at room temperature for 4 h. All of six samples were then mixed together, dried in a SpeedVac, then dissolved again in a SCX (strong ion exchange) loading buffer (10 mM KH_2PO_4 , pH = 3.0, 25% acetonitrile). This sample was loaded onto a SCX column, washed, and eluted with 500 μ L elution buffer (10 mM KH_2PO_4 , pH = 3.0, 400 mM KCl, 25% acetonitrile). The elution was dried in SpeedVac and dissolved in 100 μ L 5% acetonitrile. 4 μ L of labeled peptide sample was injected into a capillary reverse phase HPLC (gradient, 5% acetonitrile, 0.1% TFA to 50% acetonitrile, 0.1% TFA in 60 min; column, Agilent Zorbax C18, 5 μ m, 150 \times 0.5 mm; flow rate, 15 μ L/min) and each fraction was analyzed with ABI-4700 MALDI-MS/MS.

3.2. Isotope purity of DiART reagents

The isotope purity of DiART reagents was determined as described previously⁵. These numbers were included in a Mascot server configuration file in order to get correct quantitation.

Table-2. Isotope purity

$\Delta(M/Z)$	-2	-1	0	+1	+2
DiART-114	0.000	0.000	0.925	0.075	0.000
DiART-115	0.000	0.065	0.842	0.093	0.000
DiART-116	0.000	0.076	0.840	0.084	0.000
DiART-117	0.000	0.116	0.815	0.069	0.000
DiART-118	0.000	0.000	0.914	0.086	0.000
DiART-119	0.000	0.092	0.821	0.087	0.000

3.3. Compatibility of DiART reagents with custom-configured Mascot sever

Only two configuration files in Mascot sever (version 2.2 only) need to be updated to make the server compatible with DiART-based protein identification and quantitation. These two files (unimod.xml and quantitation.xml) will be distributed freely upon request. A result of Mascot searching is attached as an example.

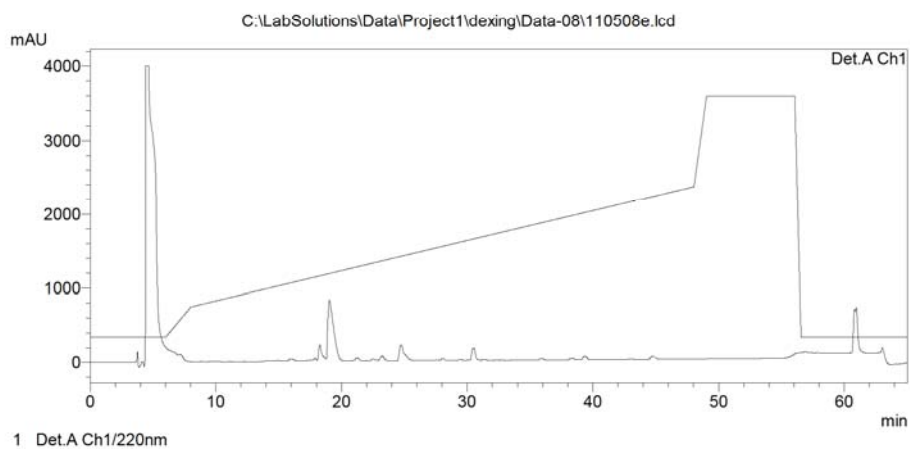
4. References

- 1) Murphy, R.F.; Douglas, A.J.; Walker, B. *US Patent* 4997950, **1991**.
- 2) Erickson, B.W. *US Patent* 4515920, **1985**.
- 3) Nakamura, M.; Yamaguchi, M.; Sakai, O.; Inoue, J. *Bioorg. Med. Chem.* **2003**, 11, 1371–1379.
- 4) Hanai, K.; Kuwae, A. *J. Labelled Comp. Rad.*, **1988**, 25, 217-224.
- 5) Zeng, D.; Li, S.W. *Bioorg. Med. Chem. Lett.*, **2009**, 19, 2059-2061.

==== Shimadzu LCsolution Analysis Report ====

C:\LabSolutions\Data\Project1\dexing\Data-08\110508e.lcd
Acquired by : Admin
Sample Name : 1
Sample ID :
Tray# : 1
Vial # : 2
Injection Volume : 100 uL
Data File Name : 110508e.lcd
Method File Name : 40min-philic.lcm
Batch File Name : 050908.lcb
Report File Name : Default.lcr
Data Acquired : 2008-11-5 22:10:13
Data Processed : 2008-11-5 23:15:15

<Chromatogram>



C:\LabSolutions\Data\Project1\dexing\Data-08\110508e.lcd

Figure 1

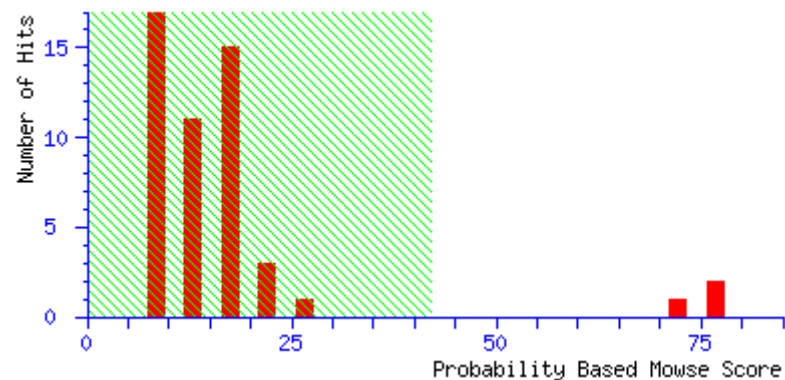
MASCOT SCIENCE Mascot Search Results

Supports the publication of Mascot search results
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User : Mascot Daemon
Email : daemon@localhost
Search title : Submitted from DiART-New by Mascot Daemon on LIW-XP
MS data file : E:\Laboratory\Projects\Proteomics\DiART\NewMultiFile2.mgf
Database : MSDB 20060831 (3239079 sequences; 1079594700 residues)
Quantitation : DiART6plex [method details](#)
 : Omic Biosystems DiART 6-plex reagent
Timestamp : 25 Mar 2009 at 02:17:26 GMT
Protein hits : 115/114 116/114 117/114 118/114 119/114
 0.996 1.022 1.056 1.095 0.910 [CATA_BOVIN](#) Catalase (EC 1.11.1.6).- Bos taurus (Bovine).
 --- --- --- --- --- [OACH](#) ovalbumin [validated] - chicken
 1.017 1.053 1.086 1.142 1.044 [ABBOS](#) serum albumin precursor [validated] - bovine

Probability Based Mowse Score

Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event.
 Individual ions scores > 42 indicate identity or extensive homology ($p < 0.1$).
 Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As Peptide Summary

[Help](#)

Significance threshold $p < 0.1$

Max. number of hits AUTO

Standard scoring MudPIT scoring Ions score or expect cut-off 0

Show sub-sets 0

Show pop-ups Suppress pop-ups Sort unassigned Decreasing Score

Require bold red

Primary type Weighted Normalisation Median ratio [Help](#)
 Supplemental material (ESI) for Chemical Communications
 This journal is (c) The Royal Society of Chemistry 2009
 Report peptide ratios Outlier removal Automatic Min. # peptides 1
 Min. precursor charge 1 Peptide threshold Minimum score of 15

Error tolerant

1. [CATA_BOVIN](#) **Mass:** 66273 **Score:** 77 **Queries matched:** 3
 Catalase (EC 1.11.1.6).- Bos taurus (Bovine).

Check to include this hit in error tolerant search or archive report

Quantitation:	Ratio	Weighted	N	SD(geo)
	115/114	0.996	3	1.073
	116/114	1.022	3	1.039
	117/114	1.056	3	NN
	118/114	1.095	3	1.061
	119/114	0.910	3	1.060

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	115/114	116/114	117/114	118/114	119/114	Peptide
<input checked="" type="checkbox"/> 1	1173.7300	1172.7227	1172.7260	-0.0033	0	31	1.8	1	0.901	0.950	1.170	1.024	0.818	K.LNSLTVGPR.G
<input checked="" type="checkbox"/> 2	1263.6600	1262.6527	1262.6751	-0.0223	0	18	48	1	1.076	1.066	1.000	1.162	0.973	R.THFSGDVQR.F
<input checked="" type="checkbox"/> 4	1502.8000	1501.7927	1501.7908	0.0019	0	30	1.7	1	0.889	1.015	1.000	0.977	0.875	R.LAHEDPDYGLR.D

Proteins matching the same set of peptides:

- [CSBO](#) **Mass:** 63859 **Score:** 77 **Queries matched:** 3
catalase (EC 1.11.1.6) [validated] - bovine
- [4BLCA](#) **Mass:** 62642 **Score:** 77 **Queries matched:** 3
catalase (EC 1.11.1.6), chain A - bovine
- [8CATA](#) **Mass:** 62513 **Score:** 77 **Queries matched:** 3
catalase (EC 1.11.1.6), chain A - bovine
- [AAI03067](#) **Mass:** 66404 **Score:** 77 **Queries matched:** 3
BC103066 NID: - Bos taurus

2. [OACH](#) **Mass:** 47756 **Score:** 75 **Queries matched:** 1
 ovalbumin [validated] - chicken

Check to include this hit in error tolerant search or archive report

Quantitation:	Ratio	Weighted	N	SD(geo)
	115/114	---	1	---
	116/114	---	1	---

117/114 --- 1 ---
 Supplementary Material (ESI) for Chemical Communications
 This journal is (c) The Royal Society of Chemistry 2009
 117/114 --- 1 ---

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	115/114	116/114	117/114	118/114	119/114	Peptide	
<input checked="" type="checkbox"/>	<u>6</u>	1904.9800	1903.9727	1904.0135	-0.0408	0	75	4e-005	1	1.028	0.985	0.964	0.877	1.201	R.GGLEPINFQTAADQAR.E

Proteins matching the same set of peptides:

[OVAL_CHICK](#) Mass: 47625 Score: 75 Queries matched: 1
 Ovalbumin (Plakalbumin) (Allergen Gal d 2) (Gal d II).- Gallus gallus (Chicken).
[IOVAB2](#) Mass: 37887 Score: 75 Queries matched: 1
 ovalbumin, chain B, fragment 2 - chicken
[IOVAC1](#) Mass: 42648 Score: 75 Queries matched: 1
 ovalbumin, chain C, fragment 1 - chicken
[CAA23681](#) Mass: 19424 Score: 75 Queries matched: 1
 GGALB1 NID: - Gallus gallus
[CAA23682](#) Mass: 47786 Score: 75 Queries matched: 1
 GGALB2 NID: - Gallus gallus
[CAC28424](#) Mass: 116079 Score: 75 Queries matched: 1
 Sequence 20 from Patent WO0104344.- Cloning vector pINT1.
[CAC28434](#) Mass: 50198 Score: 75 Queries matched: 1
 Sequence 54 from Patent WO0104344.- Cloning vector pINT1.
[AAO43266](#) Mass: 47780 Score: 75 Queries matched: 1
 AY223553 NID: - Gallus gallus

3. [ABBOS](#) Mass: 84469 Score: 73 Queries matched: 2
 serum albumin precursor [validated] - bovine

Check to include this hit in error tolerant search or archive report

Quantitation:	Ratio	Weighted	N	SD(geo)
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	116/114	1.053	2	1.169
	117/114	1.086	2	1.257
	118/114	1.142	2	1.194
	119/114	1.044	2	1.026

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	115/114	116/114	117/114	118/114	119/114	Peptide	
<input checked="" type="checkbox"/>	<u>3</u>	1356.8200	1355.8127	1355.8426	-0.0299	0	39	0.25	1	0.973	0.860	0.802	0.905	1.078	K.AEFVEVTK.L
<input checked="" type="checkbox"/>	<u>5</u>	1502.8200	1501.8127	1501.7961	0.0166	0	34	0.71	1	1.038	1.145	1.220	1.254	1.028	K.QNCDQFEK.L

Proteins matching the same set of peptides:

[Q3I349_BOSIN](#) Mass: 65911 Score: 73 Queries matched: 2

Serum albumin (Fragment).- Bos indicus (Zebu).

[AA10248](#) **Mass:** 84452 **Score:** 73 **Queries matched:** 2
Supplementary Material (ESI) for Chemical Communications
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BC102742 NID: - Bos taurus

[AAN17824](#) **Mass:** 84522 **Score:** 73 **Queries matched:** 2

AF542068 NID: - Bos taurus

[AAA51411](#) **Mass:** 84492 **Score:** 73 **Queries matched:** 2

BOVALBUMIN NID: - Bos taurus

Search Parameters

Type of search : MS/MS Ion Search
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C),DiART6plex (K),DiART6plex (N-term),DiART6plex (K),DiART6plex (N-term)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 0.8 Da
Fragment Mass Tolerance: ± 0.5 Da
Max Missed Cleavages : 0
Instrument type : Default
Number of queries : 6

Mascot: <http://www.matrixscience.com/>